Universidad Autónoma de Madrid
Facultad de Ciencias
Departamento de Biología Molecular

Identification and validation of novel targeted therapies against epithelial cancer stem cells

Patrick Christian Hermann

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Degree in Medicine

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CERTIFICA:

Que la tesis doctoral titulada "***Identification and validation of novel targeted therapies against epithelial cancer stem cells***", ha sido realizada en el Centro Nacional de investigaciones Oncológicas y tutelada en el Departamento de Biología Molecular de la Universidad Autónoma de Madrid.

La tesis realizada por Patrick Christian Hermann reúne todas las condiciones requeridas por la legislación vigente y la originalidad y calidad científica para poder ser presentada y defendida con el fin de optar al grado de Doctor.

Y para que conste donde proceda, firmo el presente certificado.
Madrid, a 25 de marzo de 2013

Prof. Dr. Christopher Heeschen

TO MY LOVING FAMILY

I want to express my deepest gratitude to my mentor, Prof. Dr. Christopher Heeschen. You have opened my eyes for the beauty and the rewards that research can mean. You have had the patience to guide me from my first steps in the lab in Munich up until today. Thank you for being a boss and a friend at the same time. Thank you for trusting in me, for giving me exciting and challenging projects, and for giving me the opportunity to work with you!

To the Heeschen Lab, thank you all for the times we have shared together. Working together with each and every one of you has been a wonderful and intense experience, and I wish you all the best in your careers and your future endeavours.

Thank you very much to all the interesting and wonderful people I have met at the CNIO, and especially for the friends I have made here. You have been a constant source of support, and it would have been impossible for me to enjoy all the ups and master the downs of the last years without you all.

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Natürlich bedanke ich mich auch und insbesondere meinen Eltern und meinen Geschwistern. Tausend Dank für eure Geduld, eure Liebe und eure Fürsorge. Und auch dafür, dass ihr es mir ermöglicht habt, meinen Weg auch hier in Spanien weiter zu gehen. Ohne euch hätte ich es nie geschafft!

THANK YOU

DANKE

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LIST OF ABBREVIATIONS

CSC	Cancer Stem Cells
CD133	Cluster of differentiation 133
CXCR4	C-X-C chemokine receptor 4
CCR7	C-C chemokine receptor type 7
SDF-1	Stromal-Derived Factor 1
ATR	Ataxia Telangiectasia- and Rad3-Related
ATM	Ataxia Telangiectasia mutated
PDAC	Pancreatic ductal adenocarcinoma
Alk	Activin Receptor-Like Kinase
Gem	Gemcitabine
mTOR	mammalian target of Rapamycin
SHH	Sonic hedgehog
FOLFIRINOX	Combination chemotherapy consisting of folic acid, 5-FU, Irionotecan, and Oxaliplatin
5-FU	5-Fluoro-Uracil
NOD/SCID	Non-obese/severe combined immunodeficiency
TGF-β	Transforming growth factor beta
ABCG2	ATP-binding cassette sub-family G member 2
MDR1	Multidrug resistance protein 1
APC	Adenomatous polyposis coli
FAP	Familial adenomatous polyposis
HNPCC	Hereditary non-polyposis colorectal cancer
EGF	Epithelial growth factor
EpCAM	Epithelial cell adhesion molecule
EMT	Epithelial-to-mesenchymal transition
GEMM	genetically engineered mouse model
PEG	Poly-Ethylene-Glycol
ICL	Interstrand crosslinking

SUMMARY

In 2007, we and another group in the US showed for the first time that human pancreatic cancers contain so-called cancer stem cells (CSCs), which express the stem cell marker CD133, which are exclusively responsible for tumourigenicity, and which are highly resistant to standard chemotherapy (Hermann et al., 2007, Li et al., 2007). Specifically, we have provided conclusive evidence that these highly tumourigenic cancer stem cells represent the “root” of the tumour due to their ability to generate all the different lineages of cells that comprise a tumour. Most importantly, we have shown for the first time that cancer stem cells are heterogeneous, and that a specific subset of CSCs expressing the chemokine receptor CXCR4 is exclusively responsible for metastatic spread of pancreatic cancer. Furthermore, the CD133+ cancer stem cells are also highly resistant to chemotherapy and irradiation, they represent an intriguing new target for therapeutic intervention.

Therefore, after comprehensively studying their molecular characteristics and defining distinct cancer stem cell-related features (such as developmental pathway activity, DNA damage response etc.), we subsequently designed several projects investigating the role of cancer stem cells in pancreatic and colorectal cancer with respect to their contribution to tumour growth and therapy resistance. More importantly, however, we were interested in elucidating the potential of cancer stem cells as potential targets for therapy. Therefore, these studies were directed towards the development of novel targeted therapies against cancer stem cells, and to investigate the influence of CSC elimination on clinical outcomes such as long-term survival in mouse models of primary pancreatic or colon cancer. With the presented studies, we are now proposing three novel targeted therapies for pancreatic and colorectal cancers to get one step closer to finally overcoming these devastating diseases.

RESUMEN

En 2007, ha sido mostrado por primera vez, tanto por nuestro equipo como por otro en los Estados Unidos, que los cánceres pancreáticos humanos contienen células troncales cancerígenas (CSC, en inglés), las cuales expresan el marcador CD133. Las células troncales cancerígenas han sido descritas por ser exclusivamente tumorigénicas, así como también altamente quimioresistentes. De manera extensiva, hemos proporcionado evidencias conclusivas que estas células troncales cancerígenas son altamente tumorigénicas y representan la raíz del tumour debido a su habilidad de generar todos los diferentes linajes celulares que conforman un tumor. Lo más importante es que hemos mostrado por primera vez que las células troncales cancerígenas son heterogéneas, y que una población distinta que expresa el receptor CXCR4 es exclusivamente responsable para la metástasis de canceres del páncreas. Siendo, las células troncales cancerígenas son altamente resistentes a la quimioterapia y radioterapia, por tanto, representan una nueva diana terapéutica.

Por lo tanto, después estudiar comprensivamente sus características moleculares y definir sus propiedades (respuesta daño AND etc.) posteriormente diseñamos distintos proyectos investigando el rol de las células troncales en páncreas y cáncer colorectal respecto a su contribución en el crecimiento tumoral y resistencia a la quimioterapia. Sin embargo, aun mas importante, estábamos interesados en mostrar el potencial de las células troncales cancerígenas como una posible diana terapéutica.
Por tanto, el objetivo de estos estudios fueron el desarrollo de nuevas dianas terapéuticas contra las células troncales cancerígenas, así como el resultado de su eliminación, midiendo la supervivencia en modelos animales de cáncer primario de páncreas o cáncer colorectal. Con estos estudios, estamos proponiendo tres nuevas dianas terapéuticas para cáncer pancreático y colorectal y así acercarnos un paso hacia adelante para finamente poder avanzar contra estos tipos de canceres tan devastadores.

INTRODUCTION

Solid tumours

Despite intense research efforts, the death toll of oncological diseases is steadily on the rise in Western countries, second only to deaths caused by cardiovascular diseases. Among all cancers, solid tumours represent the major cancer burden, and cancers arising in epithelial tissues such as breast, lung, colon, prostate and ovary constitute approximately 80% of all solid cancers. Whereas other tumour entities such as glioblastoma multiforme and pancreatic ductal adenocarcinoma are considerably less frequent, they constitute a major health risk due to their extraordinarily high mortality rates (Jemal, 2008). Tumours are generally assessed clinically at the gross level by histology and by expression of specific markers. In combination with gene expression analysis, this has lead to the definition of distinct tumour subtypes. The cellular origins of most solid tumours still remains unknown in most cases, but it is hypothesized that different subtypes correspond to distinct cells of origin at the time of tumour initiation. In addition to different tumour subtypes, cells within the tumour population frequently also exhibit functional diversity termed tumour heterogeneity (Heppner and Miller, 1983), with some cells exhibiting high proliferative and differentiating capacities.

Colorectal Cancer

Colorectal cancers are the third most frequent cancers in men as well as in women with an expected 101,340 cases of colon and 39,870 cases of rectal cancer to occur in 2011, and therefore represent a major challenge for the healthcare systems in Western countries. Colorectal cancer accounts for approximately 9% of cancer related deaths (Cancer Facts & Figures 2011). Risk factors associated with colorectal cancers are genetic pre-dispositions such as familial adenomatous polyposis (FAP) and Lynch-Syndrome (hereditary non-polypous colon cancer syndrome, HNPCC), but also age (over 40 years of age, the incidence of colorectal cancer doubles every 10 years), fat- and meat-rich diets, smoking, and alcohol intake.

These cancers arise from dysplastic epithelium, usually from colon adenomas after a sequence of genetic alteration events that were first described by Vogelstein and colleagues in 1990 (Fearon and Vogelstein, 1990). During this progression from dysplastic adenomas to full-fledged colon cancer, the loss of the APC tumour suppressor gene in normal epithelium constitutes the initiating event. Familial adenomatous polyposis (FAP) is a hereditary, autosomal dominant colorectal cancer

syndrome, which results from a mutation of APC, which can be found in 50% of the patients. Physiologically, APC is part of a protein complex binding to ß-catenin in the cytoplasm, resulting in the inhibition of its nuclear translocation. Mutation or loss of APC therefore results in ß-catenin translocation and the subsequent activation of transcription factors such as c-myc and Cyclin D1, which have been shown to be crucial players in proliferation, differentiation, migration, and apoptosis. Interestingly, another consequence of APC loss is an increased activity of the Wnt (wingless in drosophila) pathway, a signaling pathway strongly associated with stemness and self-renewal in the colon. Recent evidence even suggests that FAP may be a stem cell disease, showing an increase in the stem cell population at the bottom of intestinal crypts (Boman et al., 2008), pointing to a potentially essential role of the stem cell population during the progression process.

As the next step during progression, a mutation in the K-Ras gene leads to a constitutively active K-Ras protein, and to subsequent increase in proliferation and increased cell survival. These changes result in type II adenomas in patients, which are 1-2 cm large, and display medium-grade dysplasias. After the additional mutation or loss of the DCC tumour suppressor gene, type III Adenomas (> 2cm in size with high-grade dysplasia) can be observed in patients. The final step for the progression from high-grade adenomas to malignant carcinoma is the mutation or loss of the tumour suppressor p53. The progression to cancer is thought to take approximately 10 years, and while it is not obligatory, once a critical number of genetic changes have occurred, malignant progression is very likely to take place (**Figure 1**).

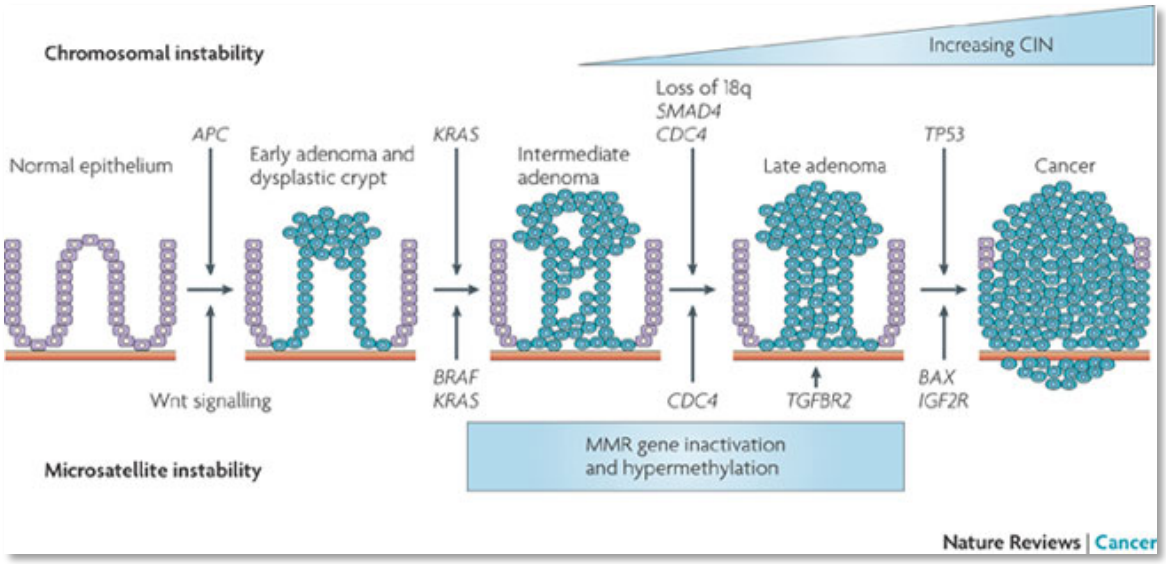

Figure1: Genetic progression model of colorectal cancer, modified after Fearon and Vogelstein. From: (Walther et al., 2009)

There has been great progress in the treatment of colon cancer over the last decades (**Table 1**). Fortunately, the mortality rates for colorectal cancer have declined significantly throughout the last 20 years. Since 1998, the rate has declined by 2.8% per year in men and by 2.7% per year in women (Cancer Facts & Figures 2011). This positive development is mainly attributed significant improvements in early detection and treatment. In clinical practice a marked shift can be observed with regards to screening methods for colorectal cancers. Fecal occult blood testing (FOBT) is an easy-to-use and low-priced diagnostic tool, which detects blood in human stool samples as a consequence of tumour bleeding. However, if the amount of blood in the stool sample is low (usually <20ml/d), tumours may remain undetected by this test. Even more importantly, however, this test is not useful for detection of early lesions such as colorectal adenomas, since they rarely bleed. Thus, while this test is easy & cheap to perform, it may not be the best option for the early detection of colorectal tumours.

In order to detect early and late neoplastic lesions in the colon and rectum, screening by endoscopy remains the gold standard. While this is an invasive and complex screening tool, it yields the best results by far, with the additional advantage of being able to take biopsies in situ during the colonoscopy. This will allow for detailed histological analysis of suspicious tissue in the very early stages of malignant transformation. Therefore, only repeated routine colonoscopy will lead to early diagnosis with high curative potential.

Surgery is the most common treatment for colorectal cancer, and may be curative for localized tumours. In advanced disease, different approaches have been tested, involving neoadjuvant or adjuvant chemotherapy (most frequently combination therapies with 5FU and/or platin-containing chemotherapies like oxaliplatin) and/or radiation. Furthermore, several targeted therapies are emerging as promising additions to the therapeutic regimen: the monoclonal antibody bevacizumab (Avastin), which binds to vascular endothelial growth factor (VEGF-A) and thus blocks the angiogenic properties of the tumour. Furthermore, two antibodies against the Epidermal Growth Factor Receptor (EGFR) have been approved by the FDA for the treatment of metastatic colorectal cancer.

However, while the prognosis of patients suffering from colorectal cancer keeps increasing, a large percentage of patients, especially with advanced metastatic cancer will still succumb to the disease. Therefore new the further development of new therapies, and the identification of new therapeutic targets is of great importance to this day.

Trends in 5-year Relative Survival Rates* (%) by Race and Year of Diagnosis, US, 1975-2006

	All races			White			African American		
	1975-77	1984-86	1999-2006	1975-77	1984-86	1999-2006	1975-77	1984-86	1999-2006
All sites	50	54	68†	51	55	69†	40	41	59†
Brain	24	29	36†	23	28	35†	27	32	41†
Breast (female)	75	79	90†	76	81	91†	62	65	78†
Colon	52	59	66†	52	60	67†	47	50	55†
Esophagus	5	10	19†	6	11	20†	3	9	13†
Hodgkin lymphoma	74	80	87†	74	80	88†	71	75	82†
Kidney	51	56	70†	51	56	70†	50	54	67†
Larynx	67	66	63†	68	68	65	59	53	49†
Leukemia	36	42	55†	36	43	56†	34	34	47†
Liver & bile duct	4	6	14†	4	6	14†	2	5	10†
Lung & bronchus	13	13	16†	13	14	17†	12	11	13†
Melanoma of the skin	83	87	93†	83	87	93†	60‡	70§	74‡
Myeloma	26	29	39†	26	27	39†	31	32	38†
Non-Hodgkin lymphoma	48	53	69†	49	54	71†	49	48	60†
Oral cavity & pharynx	53	55	63†	55	57	65†	36	36	45†
Ovary	37	40	45†	37	39	45†	43	41	37
Pancreas	3	3	6†	3	3	6†	2	5	5†
Prostate	69	76	100†	70	78	100†	61	66	97†
Rectum	49	57	69†	50	58	70†	45	46	60†
Stomach	16	18	27†	15	18	26†	16	20	26†
Testis	83	93	96†	83	93	97†	73‡#	87‡	87
Thyroid	93	94	97†	93	94	98†	91	90	95
Urinary bladder	74	78	81†	75	79	82†	51	61	66†
Uterine cervix	70	68	71	71	70	73	65	59	64
Uterine corpus	88	84	84†	89	85	86†	61	58	61

*Survival rates are adjusted for normal life expectancy and are based on cases diagnosed in the SEER 9 areas from 1975-77, 1984-86, 1999 to 2006, and followed through 2007. †The difference in rates between 1975-1977 and 1999-2006 is statistically significant ($p < 0.05$). ‡The standard error of the survival rate is between 5 and 10 percentage points. §The standard error of the survival rate is greater than 10 percentage points. #Survival rate is for 1978-1980.

Source: Altekruse SF, Kosary CL, Krapcho M, et al (eds.). *SEER Cancer Statistics Review, 1975-2007*, National Cancer Institute, Bethesda, MD, seer.cancer.gov/csr/1975_2007/, 2010.

Table 1: 5-year survival rates of colorectal cancer.

Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and deadly diseases, making it the fourth most frequent cause for death due to cancer. Full-blown pancreatic cancers have a ductal morphology, and it has been shown that tumours stem from neoplastic lesions (pancreatic intraepithelial neoplasias (PanIN)) (Feldmann et al., 2007a). Pancreatic cancers are thought to follow a progression model from low-grade (PanIN 1A and PanIN 1B) to high-grade (PanIN 2 and PanIN 3) lesions, before

becoming fully malignant PDAC (**Figure 2A and B**). This progression to invasive pancreatic cancer occurs due to the accumulation of key genetic alterations such as telomere shortening, activating mutations of the KRAS oncogene, inactivation of tumour-suppressor genes such as CDKN2A, TP53, and SMAD4 (**Figure 2C**). The importance and sequence of these genetic alterations in pancreatic tumour development is supported by studies in genetically engineered mouse models (GEMMs), in which activating mutations of KRAS with concomitant inactivation of Trp53 or Cdkn2A/Ink4A results in the development of pancreatic cancer that closely mirrors the human disease (Hingorani et al., 2005, Guerra et al., 2007, Bardeesy et al., 2006).

Altogether, while genetic inter-patient diversity in fully developed pancreatic cancers is extremely high, the key initiating mutations are extremely frequent: ~90% of the tumours have activating KRAS mutations, resulting in aberrant activation of proliferative and survival signaling pathways. 90-95% of PDACs have inactivating mutations of CDKN2A, and up to 75% of pancreatic cancers present with mutated TP53 (Maitra and Hruban, 2008).

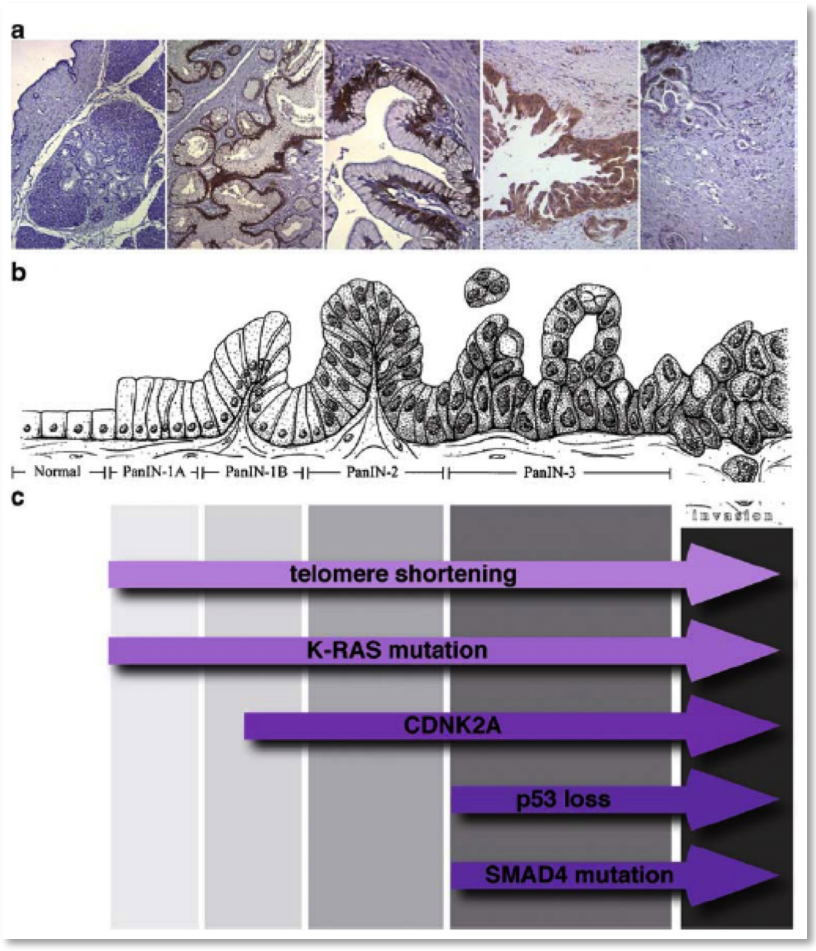

Figure 2: Key mutations and histological changes in pancreatic adenocarcinoma. From: (Mihaljevic et al., 2010)

Pancreatic adenocarcinoma is the fourth most frequent cause of cancer-related deaths. Despite its moderate incidence compared with other solid cancers, it has an exceptionally high mortality rate (Jemal et al., 2010). This is due to a combination of late diagnosis, lacking early symptoms, frequent impossibility of resecting the primary tumour, early and extensive metastasis, and high resistance to current treatments. Despite intense research and significant advances that have been achieved in the understanding of the mechanisms underlying pancreatic cancer, these advances have had only modest relevance for clinical treatment. In this respect only very little progress has been made since the introduction of the nucleoside-analogue gemcitabine in the late 1990s, which improved clinical response mainly in terms of pain reduction and loss of weight (Burris et al., 1997). Gemcitabine still remains the first-line chemotherapy for patients suffering from pancreatic cancer. However, the overall prognosis of pancreatic cancer patients remains extremely poor with the 5-year survival rate being only 1–4% (**Table 2**), and a median survival time of 4–6 months. Virtually all targeted therapies so far have failed to improve the miserable prognosis of patients with PDAC. Also the recent approval of Erlotinib, an inhibitor of the EGF-Receptor, into the therapeutic regimen resulted only in a minor prolongation of patients' survival (Moore et al., 2007). New and more promising combination therapies are currently being evaluated, as in the case of a quadruple combination of different chemotherapies (FOLFIRINOX) (Conroy et al., 2011). While the results of this study are very promising, it has to be kept in mind that the patients enrolled were a very well-selected group of patients with a high performance status. Additional trials using conventional cytotoxic approaches in combination with advanced delivery techniques such as Paclitaxel (Von Hoff et al., 2011) or in combination with stroma-targeting agents (i.e. the Infinity trial using the smoothened inhibitor IPI-926) are currently underway and bear the potential to significantly enhance response rates in patients with PDAC.

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Table 2: 5-year survival rates of pancreatic cancer.

Cancer Stem Cells and tumour heterogeneity

The fundamental cellular mechanisms underlying tumour heterogeneity are subject of intense research activities. As early as 1961, rather extraordinary studies by Southam and Brunschwig provided first evidence for heterogeneity also in tumourigenicity by autologous transplantation of malignant cells from patients with different carcinomas into subcutaneous tissue (Southam and Brunschwig, 1961). Intriguingly, the smallest inoculation resulting in transplant growth was 10^6 cells suggesting that a large number of viable cells is necessary to promote tumour growth. While it is possible that there are growth-inhibiting factors that may need to be overcome by larger populations of cells or that the provision of a peculiar local milieu suitable to the growth of the transplanted cells is mandatory for engraftment, these data are also consistent with a hierarchical organization of the tumour cells. This would implicate that only subpopulations of cells are capable of engrafting consistently.

Despite these intriguing early data, it was not before 1997 when pioneering studies from John Dick's laboratory identified for the first time leukemia-initiating stem cells (Bonnet and Dick, 1997, Reya et al., 2001) followed by landmark studies in breast cancer (Al-Hajj et al., 2003) and then rapidly emerging investigations on tumour-initiating stem cells, also termed cancer stem cells (CSC), in numerous other solid tumours.

According to the cancer stem cell hypothesis, cells with certain stem cell properties are at the bottom of the tumour hierarchy, and are the only cells that can give rise to tumours in secondary recipients. These cells have the ability so self-renew, giving rise to more cancer stem cells as well as more differentiated daughter cells (**Figure 3**).

Consequently, since CSCs are the only cells that can give rise to tumours. Interestingly, we were able to show that only a subpopulation of cancer stem cells that express the chemokine receptor CXCR4 can generate liver metastasis in a mouse model of pancreatic cancer (Hermann et al., 2007). Interestingly CXCR4 is the key receptor for stromal derived factor 1 (SDF-1), a chemokine that is detectable in high levels at typical sites for metastasis such as liver, lung, brain, and lymph nodes.

The source, or cell-of-origin of these cancer stem cells is currently unknown for solid tumours, but may vary between tissue-resident stem cells, progenitor cells, and differentiated cells, depending on the tumour type. In most solid tumours, the dysregulation of the tumour microenvironment such as chronic inflammation plays an important role during carcinogenesis, as well as secreted factors from immune cells or a tumour cell niche.

While this thesis will focus on those solid tumour entities where cancer stem cells were first identified (colon, breast, brain, and pancreas), evidence for cancer stem cells using several different (surface) markers have been found and functionally investigated in most other tumours such as prostate cancer (Collins et al., 2005, Miki et al., 2007), melanoma (Fang et al., 2005) (Schatton et al., 2008), lung cancer (Kim et al., 2005) (Bertolini et al., 2009), head and neck squamous cell carcinoma (Prince et al., 2007), and liver cancer (Yang et al., 2008) (Ma et al., 2007).

The frequency of CSC in solid tumours appears to vary considerably between tumours of the same entity from almost undetectable in some tumours to highly abundant in other tumours. Currently it is not clear whether this increase in CSC numbers is indeed related to tumour progression or related to limitations of the available set of markers for their identification, emphasizing the urgent need for more definitive

CSC markers. This must be resolved by clonality studies and more extensive studies in genetically engineered mouse models covering the full spectrum of tumour progression.

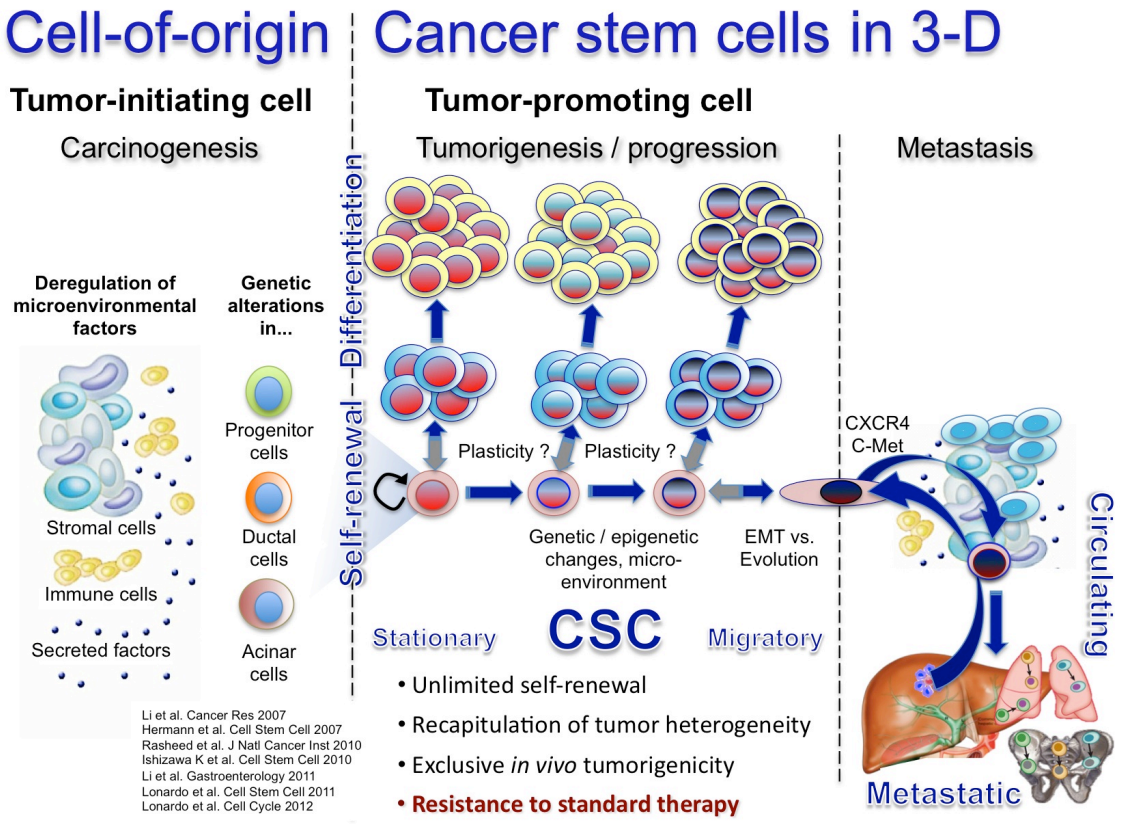

Figure 3: The cancer stem cell hypothesis in solid tumours.
Image courtesy of C. Heeschen

Colon cancer stem cells

Colon cancer is one of the few solid tumours for which the progression from a normal cell to a cancer cell is reasonably well understood. If applied to a (cancer) stem cell setting, one would hypothesize that the disease starts from one of the few stem cells at the base of the crypt. These have recently been identified by Barker et al. to express Lgr5, a leucine-rich repeat with G-protein-coupled receptor 5 (Barker et al., 2007). This landmark study seems to provide us with the most specific stem cell marker for normal intestine to date, but its role in tumourigenesis still remains to be determined. Unfortunately, it has been particularly difficult to develop high-affinity antibodies against

Lgr5 so that studies for the prospective isolation of Lgr5 positive cells from human tumour samples are still lacking.

Colon CSC were first prospectively identified in 2007 by two different research groups published back-to-back in Nature using CD133 (Ricci-Vitiani et al., 2007, O'Brien et al., 2007). In contrast, a third later study by Dalerba et al. used a combination of CD44 and EpCAM, and then identified CD166 as an additional putative CSC marker generating more consistent data as compared to CD133 (Dalerba et al., 2007). In all three studies, xenotransplantation of the isolated cells into immunocompromised mice not only led to engraftment of the investigated cells in far lower numbers than the negative cells in serial transplantation studies, but the implanted cells were also able to recapitulate the original tumour on histological levels. However, Shmelkov et al. later demonstrated that CD133 expression in colon cancer may not be restricted to CSC at all (Shmelkov et al., 2008). They claimed CD133 to be expressed on colon cancer cells regardless of their differentiation state, and demonstrated that isolated $CD133^{-}$ cells were at least as capable of giving rise to tumours in NOD/SCID mice as their CD133 positive counterparts.

Only recently, CD133 was finally shown to actually change its conformation upon differentiation, which could explain the opposing results seen with different antibodies and diverse staining protocols (Kemper et al.). Importantly, the AC133 epitope decreases upon differentiation, which was not linked to a change in CD133 promoter activity, mRNA, splice variant, protein expression, or even cell surface expression of CD133. In contrast, the only observed change concerned CD133 glycosylation suggesting that CD133 is expressed on both CSC and differentiated progenies. Since this change in glycosylation alone did not affect binding of AC133, its lack of binding to differentiated cells is more likely related to differences in protein folding as a consequence of this glycosylation. Therefore, though CD133 is certainly not a perfect marker and data need to be interpreted with great caution, it has been one of the most successful markers for the identification of CSC in various tumour entities to date.

Pancreatic cancer stem cells

First evidence for a distinct CSC population in pancreatic cancer, one of the most lethal cancers, was provided by Li and colleagues (Li et al., 2007). The authors identified a highly tumourigenic $CD44^{+}CD24^{+}EpCAM^{+}$ subpopulation using a xenograft model of

immuno-compromised mice for primary human pancreatic adenocarcinoma. Only these cells were able to form tumours at low numbers and only these cells displayed typical stem cell features like self-renewal, activation of developmental signaling pathways, generation of differentiated progeny and the ability to recapitulate the phenotype of the parental tumour from which they were derived (Li et al., 2007). Apparently, the finding that tumourigenicity in pancreatic cancer is confined to $CD24^+$ cells is in stark contrast to the original findings in breast cancer, where only $CD24^{-/low}$ cells were tumourigenic. However, these different findings have now been extended to other tumour entities such as ovarian cancer (Cao et al.). In a second study, Hermann et al. showed that CD133 in pancreatic cancer cell lines and primary pancreatic cancers also reproducibly discriminates for cells with capacity for self-renewal, sphere formation, and, most importantly, *in vivo* tumourigenicity in secondary and tertiary recipients (Hermann et al., 2007). Not surprisingly, $CD133^+$ cells show some overlap with the $CD44^+CD24^+EpCAM^+$ subpopulation. More recently, additional markers have also been associated with pancreatic CSC. ALDH-1 has been shown to label tumourigenic cells in pancreatic (Feldmann et al., 2007b, Jimeno et al., 2009, Rasheed et al.) and breast cancer (**Table 3**). Since cell surface markers merely enrich for CSC populations, and therefore their use is controversial, functional assays like sphere-formation capacity *in vitro*, and tumourigenicity *in vivo,* are becoming even more important for the identification of CSC.

Tumour Entity	Markers	Citation
Breast cancer	$CD44^+$ $CD24^{-/low}$	(Al-Hajj et al., 2003)
	$CD133^+$	(Wright et al., 2008)
	$CD133^+$ $CXCR4^+$	(Hwang-Verslues et al., 2009)
	$ALDH\text{-}1^+$	(Ginestier et al., 2007)
	$CD49F^+$ $DLL1^{high}$ $DNER^{high}$	(Pece et al.)
Colon cancer	$CD133^+$	(O'Brien et al., 2007; Ricci-Vitiani et al., 2007)
	$EpCAM^+$ $CD44^+$ $CD166^+$	(Dalerba et al., 2007)
Glioblastoma	$CD133^+$	(Singh et al., 2004)
	$SSEA\text{-}1^+$	(Son et al., 2009)
Pancreatic cancer	$EpCAM^+$ $CD44^+$ $CD24^+$	(Li et al., 2007)
	$CD133^+$	(Hermann et al., 2007)
	$CD133^+$ $CXCR4^+$	(Hermann et al., 2007)
	$ALDH\text{-}1^+$	(Feldmann et al., 2007; Jimeno et al., 2009; Rasheed et al.)
Prostate cancer	$CD44^+$ alpha2beta1 ^{high} $CD133^+$	(Collins et al., 2005)
	$CD133^+$ $CXCR4^+$	(Miki et al., 2007)
Melanoma	$CD20^+$	(Fang et al., 2005)
	$ABCB5^+$	(Schatton et al., 2008)
Lung cancer	$Sca\text{-}1^+$, $CD45^-$, $PECAM^-$, $CD34^+$	(Kim et al., 2005)
	$CD133^+$ $CXCR4^+$	(Bertolini et al., 2009)
Head & Neck cancer	$CD44^+$ BMI-1	(Prince et al., 2007)
Liver cancer	$CD90^+$	(Yang et al., 2008)
	$CD133^+$	(Ma et al., 2007)

Table 3: Several surface markers and marker combinations have been used for the identification of cancer stem cells in solid tumours
From: (Hermann et al., 2010)

Metastasis is the major cause of death in pancreatic cancer patients and currently there is no effective treatment available for this deadly disease. However, not all cells within a tumour possess the same metastatic potential, and only a rather small subset of cells is directed through lymphatic or blood vessels toward specific secondary sites to form metastases. In order to be able to establish secondary lesions, the migrating cells would require similar features to the cells initiating the primary tumour.

For this reason CSC were proposed to represent the only cell population capable of spreading and giving rise to metastases. Indeed, Hermann et al. for the first time identified two distinct subsets of CSC based on the expression of the chemokine receptor CXCR4 in pancreatic cancer. CXCR4 is a chemokine receptor responding to chemotactic gradients of its specific ligand SDF-1 that was originally found to be responsible for leukocyte and hematopoietic progenitor cell homing. Emerging evidence suggests that CXCR4 plays a pivotal role in the metastatic process of different tumour entities towards a gradient of SDF-1, which is highly expressed in secondary sites usually associated with metastasis.

In 2007, we identified a "stationary" population expressing CD133, but not CXCR4, which is responsible for the initiation and maintenance of the primary tumour, and a "migrating" and highly metastatic population characterized by co-expression of CD133 and CXCR4. Only $CD133^{+}CXCR4^{+}$ cells had metastatic potential, while depletion of the CSC population for $CD133^{+}CXCR4^{+}$ cells completely abrogated the usually strong metastatic phenotype of the implanted tumours (**Figure 4**) (Hermann et al., 2007).

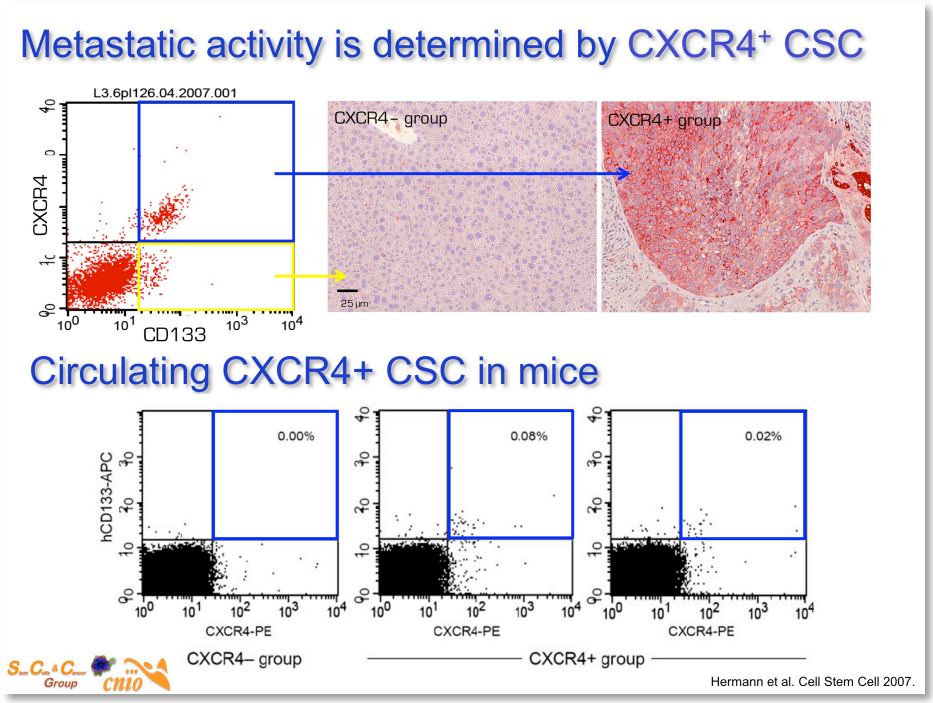

Figure 4: Identification of a metastatic subpopulation of cancer stem cells generating liver metastasis (top panel), and giving rise to circulating tumour cells (bottom panel).
From: (Hermann et al., 2007)

Consequently, pharmacological inhibition of the CXCR4 receptor by AMD3100 also prevented the metastatic activity of purified CSC. These data provide convincing evidence for a crucial role of the SDF-1/CXCR4 axis in metastasis. Since most cancers initially spread to local lymph nodes long before solid organ colonization, the lymphatic system and lymph node metastases also need to be investigated for the presence and contribution of migrating CSC. Indeed, we also found significantly higher numbers of $CD133^+CXCR4^+$ migrating CSC in patients with lymph node metastasis (pN1+), demonstrating a close clinical correlation between migrating CSC and advanced disease (Hermann et al., 2007). A different study by Nakata et al. suggested that CCR7, another chemokine receptor (also know as BLR2 or CD197), is also associated with lymph node metastasis in pancreatic cancer and, based on multivariate survival analysis, could serve as an independent prognostic factor (Nakata et al., 2008).

CSC may acquire a migrating phenotype through Epithelial-Mesenchymal-Transition (EMT) in primary tumours, because the mesenchymal phenotype is usually associated with strong migration capacity while maintaining stemness, thus allowing the production of progenies during metastasis. Recently, Weller et al. showed in pancreatic and colon cancer that the EMT-activator ZEB1 represents an important promoter of metastasis by suppressing E-cadherin. Furthermore, the stem cell phenotype was maintained by suppression of miR-200 family members that usually target stem cell factors such as Sox2 and Klf4 (Wellner et al., 2009). Together, these results suggest that in cancer the metastatic process is not random, but rather regulated by specific mechanisms related to the expression of adhesion molecules, chemokine receptors and their respective ligands.

Several studies have shown that standard therapy has limited or no significant effect on CSC, but only enriches for these population due to the elimination of more differentiated cells (Bao et al., 2006, Hermann et al., 2007, Mueller et al., 2009). For this reason it is important to identify new therapeutic approaches that can (selectively) eliminate this population and thus improve cancer treatment. It has been demonstrated consistently that the treatment with the first-line chemotherapeutic agent gemcitabine of fresh and *in vivo* expanded patient-derived pancreatic cancer cells preferentially targets the more differentiated tumour cells with a resulting enrichment of $CD133^+$ cells in which the tumourigenic population is contained. Gemcitabine treatment of nude mice bearing orthotopic human tumour xenografts is only effective to control the tumour growth and prolong survival, but does not affect CSC as the root of the tumour. The basis of

resistance to chemotherapy in this population is provided by anti-apoptotic mechanisms (Visvader and Lindeman, 2008), increased repair of DNA after damage, and by the presence of membrane transporters that pump drugs out of these cells (Goodell et al., 1996); this way the CSC population is protected from damage caused by external agents. This population shows the ability to efflux the fluorescent dye Hoechst 33342, producing a characteristic profile in flow cytometry analyses. This ability has been attributed to the expression of the transporters ABCG2 and MDR1 and has been related to tumour-initiating cells (Hirschmann-Jax et al., 2004, Ho et al., 2007), and may well be responsible for the resistance to chemotherapeutic agents and thus for the exceptional malignancy of pancreatic cancer (Zhou et al., 2008). Thus, withdrawal of gemcitabine treatment usually results in a rapid relapse of tumour growth and increased aggressiveness of the tumour.

However, these latter approaches are unlikely to eliminate the root of PDAC as they primarily target proliferative and more differentiated cells. Since the identification of exclusively tumourigenic cells in the hematopoietic system several decades ago, stem cells have changed the way we study biology and medicine. More recently it has been shown that stem cells play a decisive role not only in the generation of complex multicellular organisms but also in the initiation and propagation of solid tumours (Clarke et al., 2006), (Jordan et al., 2006). These cells with stem cell properties (therefore termed cancer stem cells; CSC) are an integral part for the perpetuation and progression of various human cancers (Al-Hajj et al., 2003) (Kim et al., 2005) (O'Brien et al., 2007) (Ricci-Vitiani et al., 2007) (Singh et al., 2004), and, according to the current consensus definition, are able to self-renew and to produce all the heterogeneous lineages of cancer cells that comprise the tumour (Clarke et al., 2006).

Increasing evidence now suggests that among several other solid malignancies, the CSC model can also be applied colon cancer. A CD133+ subpopulation of colon cancer cells derived from primary tumours was shown to be highly enriched for tumourigenic colon CSCs, capable of self-renewal and the recapitulation of the bulk tumour population (O'Brien et al., 2007, Ricci-Vitiani et al., 2007), thus demonstrating the three defining properties of cancer stem cells. Based on this demonstrated principle of CSCs as the root of the tumour and their resistance toward conventional chemotherapy (Hermann et al., 2007, Bao et al., 2006, Todaro et al., 2007), the development of

approaches aiming at the specific eradication of CSCs in colorectal tumours represents an innovative goal for improved colorectal cancer treatment.

The cancer stem cell hypothesis has also been shown to hold true for pancreatic cancer (Li et al., 2007, Hermann et al., 2007). Furthermore, it has been shown that CSCs may not only be associated with tumour growth, but also contain a subpopulation of migrating cancer stem cells that is exclusively responsible for metastatic spread to secondary tumour sites (Hermann et al., 2007). Furthermore it has been demonstrated that CSCs are highly resistant to chemotherapy (Hermann et al., 2007) as well as radiation (Bao et al., 2006), standard therapeutic approaches that preferentially target differentiated tumour cell populations, but sparing cancer stem cells.

Because these cells cannot be targeted by standard cytotoxic therapy, and at the same time possess the potential to repopulate the entire tumour (thus explaining the surprising clinical finding of relapsed disease after previous regression, CSCs offer a very challenging, but at the same time promising novel entity for the development of new therapies.

AIMS OF THE PROJECT

1. Identification of putative new targets and signaling pathways that may serve to eliminate cancer stem cells

2. Functional and molecular validation of the previously identified pathways and their significance in cancer stem cells

3. *In vitro* inhibition of the previously identified targets and evaluation of its effects on cancer stem cells

4. *In vivo* inhibition of the previously identified targets and evaluation of its effects on tumour growth, survival, and cancer stem cell populations in a clinically most relevant model of primary human pancreatic and colon cancer

RESULTS

Inhibition of Ataxia Telangiectasia- and Rad3-Related Function Abrogates the In Vitro and In Vivo Tumourigenicity of Human ColonCancer Cells Through Depletion of the CD133+ Tumour-Initiating Cell Fraction

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A very typical characteristic of cancer stem cells is a highly efficient response to DNA-damage, which is partly dependent on their more robust activation of DNA-damage checkpoint proteins, and which leads to their widely described chemo-resistance towards DNA-damaging drugs. In this part of the thesis project we established the abrogation of DNA-damage checkpoints through inhibition of the PIK kinase ATR as a therapeutic approach for the elimination of cancer stem cells in colon cancer: Using three independent model systems (chemical ATR inhibition, genetic inactivation of the ATR gene, and siRNA-mediated ATR protein depletion), we demonstrated that ATR inhibition depleted the cancer stem cell fraction of established human colon cancer cell lines as well as xenograft-derived primary colon cancer cells. This effect was attributable at least in part to apoptosis, accelerated on combined treatment with DNA-damaging chemotherapeutics, and accompanied by a drastically decreased *in vitro* and *in vivo* tumourigenicity of the remaining cells. This specific depletion of cancer stem cells was mechanistically due to the preferential activation of the ATR-dependent DNA-damage response in cancer stem cells. Our study thus illustrates a novel therapeutic approach to overcome the chemoresistance of cancer stem cells and specifically eliminate the very subpopulation of tumour cells that is exclusively responsible for tumour development, growth, and metastasis in colon cancer. Furthermore, in a second line of investigation, we have shown that the combination of ATR / CHK1 inhibition and conventional chemotherapy successfully eliminates CSCs in human colon cancer cell lines, providing a rationale for a new combination chemotherapeutic approach against colorectal cancers. Since currently no specific ATR inhibitors are available, we are collaborating with the Experimental Therapeutics Programme of the CNIO to identify and develop such compounds, which are ready to be tested in (pre-) clinical studies within the Clinical Research Programme of the CNIO and collaborating hospitals.

I contributed to the design of this study, performed the experiments together with the other authors, analyzed and interpreted the results. I also participated in the writing of the manuscript, with input from the rest of the authors and under the supervision of the thesis director Prof. Christopher Heeschen.

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Inhibition of Ataxia Telangiectasia- and Rad3-Related Function Abrogates the In Vitro and In Vivo Tumorigenicity of Human Colon Cancer Cells Through Depletion of the CD133$^+$ Tumor-Initiating Cell Fraction

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Key Words. ATR • Cancer stem cells • Colon cancer • DNA-damage response • Tumor-initiating cells

ABSTRACT

The identification of novel approaches to specifically target the DNA-damage checkpoint response in chemotherapy-resistant cancer stem cells (CSC) of solid tumors has recently attracted great interest. We show here in colon cancer cell lines and primary colon cancer cells that inhibition of checkpoint-modulating phosphoinositide 3-kinase-related (PIK) kinases preferentially depletes the chemoresistant and exclusively tumorigenic CD133$^+$ cell fraction. We observed a time- and dose-dependent disproportionally pronounced loss of CD133$^+$ cells and the consecutive lack of in vitro and in vivo tumorigenicity of the remaining cells. Depletion of CD133$^+$ cells was initiated through apoptosis of cycling CD133$^+$ cells and further substantiated through subsequent recruitment of quiescent CD133$^+$ cells into the cell cycle followed by their elimination. Models using specific PIK kinase inhibitors, somatic cell gene targeting, and RNA interference demonstrated that the observed detrimental effects of caffeine on CSC were attributable specifically to the inhibition of the PIK kinase ataxia telangiectasia- and Rad3-related (ATR). Mechanistically, phosphorylation of CHK1 checkpoint homolog (*S. pombe*; CHK1) was significantly enhanced in CD133$^+$ as compared with CD133$^-$ cells on treatment with DNA interstrand-crosslinking (ICL) agents, indicating a preferential activation of the ATR/CHK1-dependent DNA-damage response in tumorigenic CD133$^+$ cells. Consistently, the chemoresistance of CD133$^+$ cells toward DNA ICL agents was overcome through inhibition of ATR/CHK1-signaling. In conclusion, our study illustrates a novel target to eliminate the tumorigenic CD133$^+$ cell population in colon cancer and provides another rationale for the development of specific ATR-inhibitors. STEM CELLS *2011;29:418–429*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

According to the cancer stem cell (CSC) model, solid tumors may not be viewed as simple monoclonal expansions of functionally equal cancer cells. Instead, despite their clonal origin, only a fraction of tumor cells, termed CSC, "tumorigenic cells" or "tumor-initiating cells," appears to bear exclusive tumorigenicity based on functional assays of self-renewal and tumor initiation [1–3]. Increasing evidence suggests that among several other solid malignancies, the CSC model can also be applied to colon cancer. A CD133$^+$ subpopulation of colon cancer cells derived from primary lesions was shown to be highly enriched for tumorigenic colon CSC capable of

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self-renewal and recapitulation of the bulk tumor population [4, 5]. Based on the demonstrated principle of CSC as the root of the tumor and their resistance toward conventional chemotherapy [3, 6–8], the development of approaches aiming at the specific eradication of CSC in solid tumors represents an innovative goal for improved cancer treatment.

Recently, cell cycle modulation through checkpoint abrogation emerged as a promising approach in cancer therapy. In contrast to normal cells, cancer cells appear to be selectively sensitive toward treatment with inhibitors of checkpoint kinases, especially when these agents were combined with DNA interstrand-crosslinking (ICL) agents [9, 10]. Importantly, a preferential activation of the DNA-damage response (DDR), comprising both amplified DNA-damage checkpoint activation and increased repair of DNA-damage, has been described as likely mechanism of CSC drug- and irradiation-resistance in several tumor entities [11, 12]. However, the impact of checkpoint abrogation through inhibition of checkpoint kinases specifically on the CSC population in colon cancer has not yet been systematically explored. Therefore, we investigated the effects of checkpoint-modulation through inhibition of phosphoinositide 3-kinase-related (PIK) kinases specifically on the exclusively tumorigenic $CD133^+$ colon cancer cell population in multiple model systems using primary colon cancer cells and human colon cancer cell lines for a comprehensive mechanistic investigation.

Materials and Methods

Cell Lines, Primary Colon Cancer Samples, and Cell Culture

The human colon cancer cell lines DLD1, Colo320, and RKO were purchased from the American Type Culture Collection (ATCC, Wesel, Germany, www.atcc.org). COGA-12 was kindly provided by M. Ogris (Department of Pharmacy, Ludwig-Maximilian-University Munich, Germany). RKO cells harboring an inactivating deletion of *FANCC* and *FANCG*, respectively, and DLD1 cells harboring the Seckel mutation of the ataxia telangiectasia- and Rad3-related (*ATR*) gene have been described previously [13, 14]. Primary colon cancer cells for ex vivo experiments were established through subcutaneous xenografting in nude mice according to a protocol described earlier [15]. Importantly, all in vivo-expanded cell lines used in our study were transplanted as tissue, using their natural environment for expansion according to a previously established protocol [16] and analyzed during early passages: CCR004 (passage 5), CCR005 (passage 5), CCR010 (passage 2), CCR14 (passage 8–10), and CCR19 (passage 3). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal calf serum and penicillin-streptomycin (1%) and incubated at 37°C and 5% CO_2. Cells were treated with caffeine (Sigma-Aldrich, Munich, Germany, www.sigmaaldrich.com), UCN-01 (Sigma), SB-218078 (Merck KG, Darmstadt, Germany, www.merck.de), RAD001 (Novartis GmbH, Nuremberg, Germany, www.novartis.de), KU-55399 (Sigma), cisplatin (cis-diammineplatinum(II)-dichloride; Sigma).

Sphere Formation Assays

CSC spheres were cultured in DMEM-F12 (Invitrogen, Karlsruhe, Germany, www.invitrogen.com) supplemented with B-27 (Invitrogen) and basic fibroblast growth factor (bFGF) (PeproTech EC, London, U.K., www.peprotech.de). A total of 10,000 cells per milliliter were seeded in ultra-low attachment plates (Corning B.V., Schiphol-Rijk, The Netherlands, www.corning.com) to avoid cell adhesion and subsequent differentiation. Three to five days after treatment, four visual fields of at least two wells were counted. Three or more independent experiments were performed for each group. When no sphere formation occurred, the remaining single cells were kept under sphere culture conditions for up to 12 days to ensure a sufficient observation time. For primary cancer cell experiments, in vivo-expanded primary colon cancer samples from five different patients were digested with collagenase (Stem Cell Technologies, Vancouver, Canada, www.stemcell.com) for 20 minutes at 37°C. Dead cells were removed using a kit according to the manufacturer's instructions (Miltenyi, Bergisch-Gladbach, Germany, www.miltenyibiotec.com). Sphere formation capacity was assessed 3–11 days after treatment. For sphere reformation, single-cell suspensions were plated in normal sphere medium after 11 days of treatment. Spheres were defined as morphologically characteristic three-dimensional structures of approximately >35 μm, containing an average of 50 cells. According to this definition, all sphere formation experiments were evaluated by two-blinded observers (P.C.H./C.H. or M.T.M./C.H., respectively).

Animals and Transplantation of Human Colon Cancer Cells

Female Naval Medical Research Institute nude mice (NMRI-nu/nu, Janvier, Le Genest-Saint-Isle, France, www. janvier-europe.com) at 8–12 weeks were used. All animal protocols were approved by the Institutional Animal Care and Use Committee of the CNIO or by the Administrative Panel on Laboratory Animal Care (Government of Upper Bavaria, Germany). Colon cancer cells were injected under the renal capsule of anesthetized mice as described previously [4]. One day prior to cell transplantation, mice were sublethally irradiated (350 cGy). Then, single-cell suspensions were suspended in a 1:1 mixture of media and Matrigel (BD Biosciences, Heidelberg, Germany, www.bdbiosciences.com) and 5×10^5 cancer cells were injected using a PB600 repeating dispenser (Hamilton AG, Bonaduz, Switzerland, www.hamilton-ag.ch). Tumorigenicity of DLD1 $ATR^{+/+}$ cells was validated in two independent experiments ($n = 10$ mice, 100% take rate) and served as a control for all animal experiments involving DLD1 cells. Additionally, all experiments were microscopically evaluated to confirm sufficient cell grafting (through confirmation of single tumor cells or small aggregates, respectively, under the renal capsule) and to exclude macroscopically invisible small tumor formation in macroscopically tumor-negative animals. For s.c. transplantation models, single-cell suspensions containing 2×10^4 xenograft-derived primary cells were suspended as described above and implanted s.c. into both flanks of nude mice.

Flow Cytometry

For the identification and FACSorting of colon CSC, cells were stained with allophycocyanin- or phycoerythrin-labeled CD133/1 (Miltenyi) or epithelial cell adhesion molecule (EpCAM) (BD) antibodies or appropriate isotype-matched control antibodies. Dead cells were excluded using 7-amino-actinomycin D (7-AAD) (BD Biosciences) or 4',6-diamidino-2-phenylindole (Sigma). Cell cycle analysis was performed using a 5-bromo-2-deoxyuridine (BrdU) flow cytometry kit (BD) according to the manufacturer's instructions. For detection of apoptotic cells, costaining with 7AAD and Annexin V-fluorescein isothiocyanate (FITC) (BD) was performed. Caspase inhibitors Z-VAD-FMK (R&D Systems, Minneapolis, MN, www.rndsystems.com), Z-IETD-FMK (BD), and Z-LEHD-FMK (BD) were applied at 20 μM for 3 hours, followed by caffeine treatment. Flow cytometry was performed on a FACSCanto II, FACSorting on a FACSAria II

(both BD), and data were analyzed using FlowJo 9.0.2 (Ashland, OR, www.flowjo.com).

siRNA-Mediated ATR Protein-Depletion

DLD1 cells at 30%–50% confluence were transfected using oligofectamine (Invitrogen) and siRNA directed against either ATR (Hs ATR 12 HP, Qiagen, Hilden, Germany, www.qiagen.com) or CHK1 checkpoint homolog (*S. pompe*) (CHK1) (Hs CHEK1 7, HP, Qiagen) or non-coding sequences of the β-galactosidase (β-gal) gene (sense, UUAUGCCGAUC GCGUCACAUU; antisense, UGUGACGCGAUCGGCAUA AUU; Fisher, Schwerte, Germany, www.de.fishersci.com). siRNAs were used at final concentrations of 5 nM (ATR siRNA) or 50 nM (CHK1 and β-gal siRNA). After transfection for 4 hours, serum-containing medium was added. To ensure efficient long-term downregulation, siRNAs were applied repetitively (according to a previously established protocol at 48, 96, 168, 216, 264, 336, and 384 hours for ATR and at 48, 96, 168, and 216 hours for CHK1). Efficiency of ATR and CHK1 protein-depletion was evaluated using Western blotting.

Western Blotting

Western blotting was performed using standard protocols. Briefly, equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking, the membranes were incubated overnight with the primary antibody either against ATR (Santa Cruz Biotechnology, Santa Cruz, CA, www.scbt.com), pChk1 (Cell Signaling, Danvers, MA, www.cellsignal.com), β-actin or glyceraldehyde 3-phosphate dehydrogenase (Sigma), washed three times, and probed with the corresponding secondary antibodies (1:10,000; Santa Cruz) for 2 hours. Enhanced chemiluminescence detection was performed according to the manufacturer's instructions (Little Chalfont, U.K., www.gelifesciences.com).

Immunohistochemistry

For histological evaluation, tumor tissue was fixed in formalin and embedded in paraffin. Histological staining for cytokeratin 5, 6, 8, 17, 19 (Dako, Hamburg, Germany, www.dako.com), and hemalaun (Sigma) confirmed the nature of the tumors. For immunostaining, slides were incubated for 1 hour with a Ki67 antibody (Abcam, Cambridge, U.K., www.abcam.com) followed by a biotin-labeled secondary antibody (Cytomed, Baden-Baden, Germany, www.cytomed.de) and streptavidin-FITC for detection. CSC were identified by Texas Red-labeled antibodies for CD133 (Abcam). Cell nuclei were counterstained with Sytox Blue (Invitrogen). Sections were analyzed with a Leica SP5 confocal microscope.

Statistical Analyses

Results for continuous variables are presented as means ± SEM. Treatment groups were compared with the independent sample's *t* test. Pairwise multiple comparisons were performed with the one-way analysis of variance (two-sided) with Bonferroni adjustment. p values $<.05$ were considered statistically significant. All analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, www.spss.com).

Results

Tumorigenicity Is Restricted to the CD133$^+$ Cell Fraction in DLD1 Colon Cancer Cells

We first evaluated whether CD133 represents a suitable marker for the identification of tumor-initiating cells in the colon cancer cell line DLD1 as a versatile model system. Consistent with previous findings for primary human colon cancer cells [4, 5], we detected a small fraction of CD133$^+$ cells in DLD1 cells (Fig. 1A). Separation of DLD1 subsets on the basis of CD133 expression resulted in the significant enrichment of CD133$^+$ cells and the efficient negative selection of CD133$^-$ cells (Fig. 1B, second and third panel). CD133$^-$ cells, subsequently kept under adherent culture conditions for 14 days, were not capable of producing CD133$^+$ cells, whereas CD133$^+$ cells readily generated a heterogeneous population of CD133$^+$ and CD133$^-$ cells compositionally comparable with that of unsorted cells (Fig. 1B, first and fourth panel). Sphere formation capacity as a surrogate for CSC activity and in vitro tumorigenicity [5, 7] was significantly lower for CD133$^-$ cells as compared with CD133$^+$ cells (Fig. 1C). The depletion of the tumor-initiating fraction in a cancer cell population is expected to decrease the in vivo tumorigenicity of the remaining cells. Therefore, we assessed the ability of sorted CD133$^+$ and CD133$^-$ DLD1 cells to engraft and give rise to tumors when implanted under the renal capsule of sublethally irradiated athymic nude mice [4]. Although 10^4 CD133$^-$ cells did not form tumors in any of the mice, the injection of 10^4 CD133$^+$ cells resulted in macroscopic tumor lesions in all animals within 30 days (Fig. 1D). Therefore, CD133 can serve as a suitable marker for the identification of the tumorigenic cell fraction in DLD1.

Depletion of the CD133$^+$ Cell Fraction By Caffeine Treatment Abrogates In Vitro and In Vivo Tumorigenicity of Colon Cancer Cells

Treatment with caffeine, an unspecific inhibitor of checkpoint-modulating PIK kinases, at 0.2–5 mM over a time period of 21 days every 2 days dose-dependently depleted the CD133$^+$ cell fraction of DLD1 cells (Fig. 2A). This effect became statistically significant at 5 mM (Fig. 2B). Caffeine treatment of another colon cancer cell line, COGA12, had a similar effect, ruling out cell line-specific artifacts (Fig. 2C).

As sphere formation capacity serves as a surrogate marker for CSC activity and in vitro tumorigenicity of cancer cells in solid tumors [5, 8], DLD1 cells were next treated over a time period of 21 days every 2 days with caffeine at 5 mM and consecutively, sphere formation was evaluated. Although untreated control cells readily formed spheres within 3–5 days, caffeine-treated cell populations exhibited a strongly diminished sphere formation capacity (Fig. 2D).

As the depletion of the tumor-initiating CD133$^+$ cell fraction is expected to decrease the in vivo tumorigenicity of the remaining subpopulation, DLD1 cells were next treated over a time period of 21 days every 2 days with caffeine at 1 and 5 mM and then implanted under the renal capsule of nude mice. Thirty days after cell implantation, tumor take rate and tumor size were assessed (see Fig. 2E for experimental setup). We observed a dose-dependent decrease of the in vivo tumorigenicity of the cell populations pretreated with caffeine as shown by a decreased to completely absent tumor take rate (Fig. 2F). In contrast, large tumor formation was observed in all animals of the control group (Fig. 2G).

Depletion of the CD133$^+$ Cell Fraction by Caffeine Treatment Abrogates In Vitro and In Vivo Tumorigenicity of Primary Colon Cancer Cells

To generalize our findings beyond the setting of established cell lines, we expanded primary colon cancer cells from surgical tumor specimens using an in vivo xenograft model modified from Jimeno et al. [15]. Xenograft-derived tumor

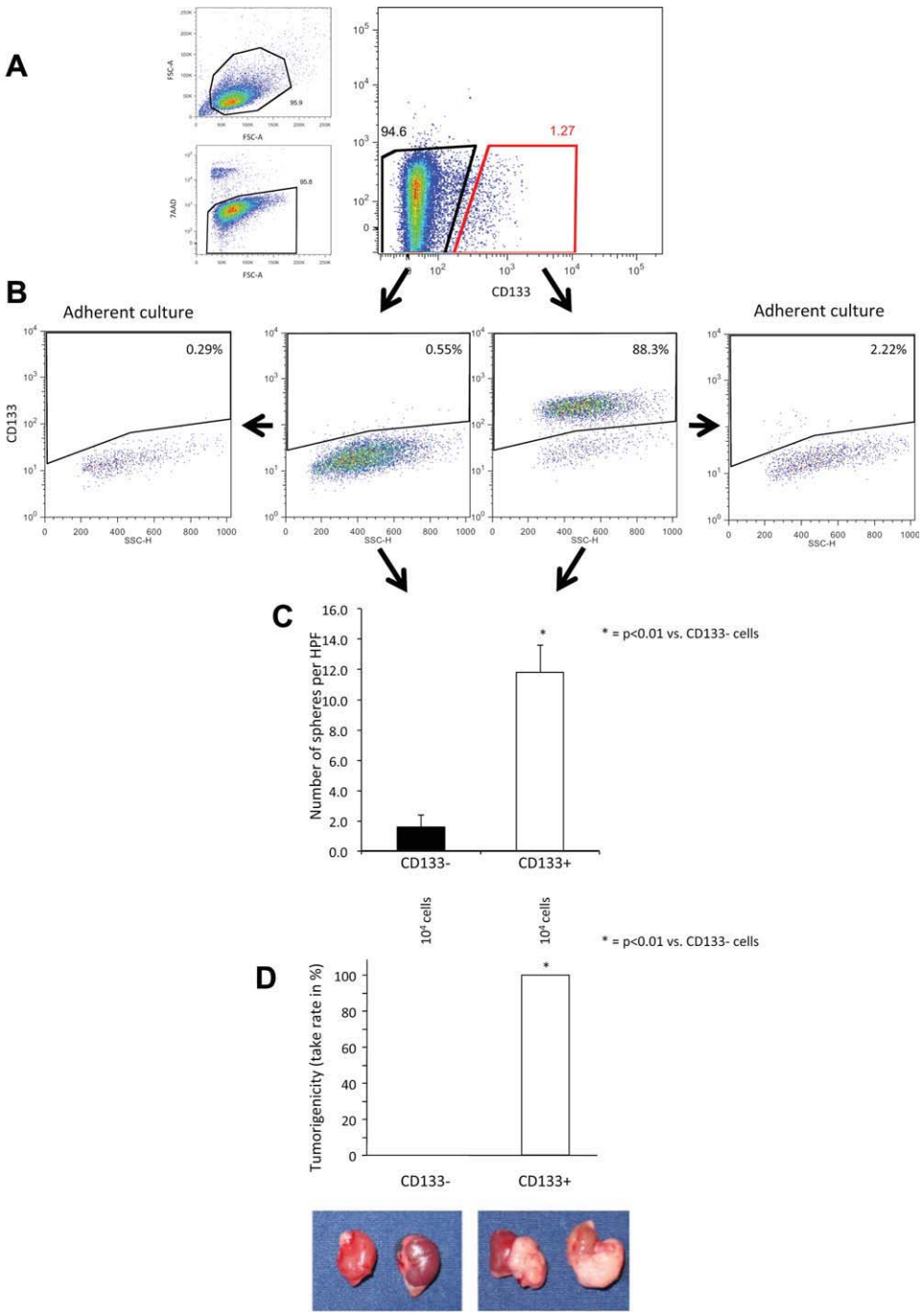

Figure 1. CD133$^+$ as a marker for the tumorigenic fraction of DLD1 colon cancer cells. CD133 expression in DLD1 colon cancer cells **(A)** before and **(B)** after FACS according to CD133 expression (second panel CD133$^-$ cells, third panel CD133$^+$ cells). Subsequent adherence culture of sorted CD133$^+$ (fourth panel) and CD133$^-$ cells (first panel). **(C):** Sphere formation capacity of CD133$^-$ and CD133$^+$ cells. **(D):** Tumor take rate after injection of 10^4 CD133$^+$ or CD133$^-$ cells, respectively, under the renal capsule of nude mice ($n = 5$ per group): Statistical evaluation (upper panel) and representative macroscopic images (lower panel). Note that the connective tissue adhering to the kidneys on the left picture represents adipose tissue. Abbreviations: 7AAD, 7-aminoactinomycin D; FSC-A, forward scatter - area; SSC-H, side scatter - height.

specimens of five patients were dissociated and single-cell suspensions investigated for surface expression of CD133 prior to and 11 days after caffeine treatment. Coexpression of EpCAM was used to discriminate tumor cells from potential contaminating endothelial or hematopoietic stem and progenitor cells. We found a significant decrease of the EpCAM$^+$ CD133$^+$ cell fraction in primary tumor cells after caffeine treatment (Fig. 3A, left panel). Importantly, we observed single viable cells, which were not clonally expanding under these conditions by the end of the treatment period (Fig. 3A, right panel), although apparently lower in numbers as compared with control cells, thus confirming a preferential targeting of the clonally expanding cells. Consistently, caffeine-treated primary colon cancer cells, although viable, demonstrated a strongly diminished sphere formation capacity as compared with control cells (Fig. 3B).

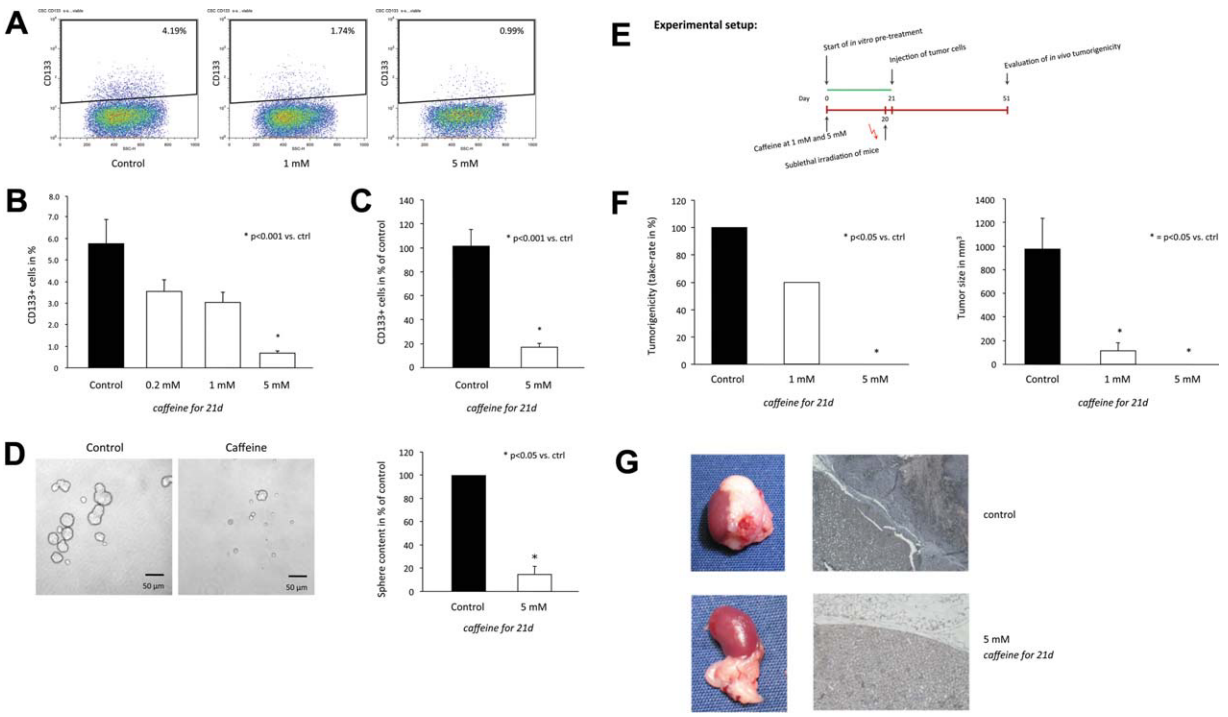

Figure 2. Dose-dependent depletion of CD133$^+$ cells after caffeine treatment and reduced in vitro and in vivo tumorigenicity of the remaining cell population: CD133$^+$ fraction of DLD1 cells treated with caffeine at the indicated concentrations for 21 days. **(A):** Representative results and **(B)** statistical evaluation ($n = 5$). **(C):** CD133$^+$ fraction of COGA12 cells treated with caffeine for 21 days ($n = 3$). **(D):** Representative images (left two panels) and statistical evaluation ($n = 3$; right panel) of sphere formation capacity of DLD1 cells treated with caffeine or control for 21 days. **(E):** Experimental setup for the in vivo experiments. **(F):** In vivo tumorigenicity, evaluated 30 days after injection of DLD1 cells under the renal capsule of nude mice: tumor take rate (left panel) and volume (right panel) after implantation of cells treated with control ($n = 10$) or caffeine at 1 mM ($n = 5$) or 5 mM ($n = 7$), respectively. **(G):** Representative macroscopic and microscopic pictures of tumor formation after injection of untreated control cells (upper panel) or cells treated with caffeine for 21 days (lower panel). Abbreviations: CSC, cancer stem cell; SSC-H, side scatter - height.

As we detected a small proportion of viable CD133$^+$ cells after caffeine treatment, representing either CD133$^+$ nontumorigenic cells or surviving tumorigenic cells, we performed sphere reformation assays after caffeine treatment. Although control cells reproducibly generated second generation spheres, no sphere formation was observed in the caffeine-pretreated cells, when cultured in caffeine-free medium (Fig. 3C). Finally, dissociated control or caffeine pretreated single-cell suspensions, derived from xenograft-derived tumor specimens from three patients, were implanted s.c. in the respective flanks of the same mice to allow direct comparisons and to exclude interindividual take rate variability. During an observation period of 100 days, we observed a reduced tumor take rate, a decreased average size of engrafted tumors, and a significantly higher event-free long-term survival in the caffeine-treated group (Fig. 3D).

Caffeine Treatment Increases Proliferation and Apoptosis of CD133$^+$ Colon Cancer Cells

To elucidate the mechanism underlying the caffeine-mediated depletion of the tumorigenic CD133$^+$ cell fraction, we compared the effects of caffeine on proliferation of and apoptosis in CD133$^+$ cells versus CD133$^-$ cells. For technical reasons, we used colon cancer cell lines as a model system for these experiments. DLD1 cells were treated over a time period of up to 7 days with caffeine at 5 mM. Consecutively, BrdU or Annexin V staining along with concomitant CD133 staining was performed to separately analyze CD133$^+$ and CD133$^-$ cells with regard to proliferation and apoptosis. In the control group, 10% of CD133$^-$ cells and 17% of CD133$^+$ cells showed an early BrdU incorporation by 2 hours, while up to 96% of CD133$^-$ cells, but only 56% of CD133$^+$ cells had incorporated BrdU by 96 hours, indicative of a quiescent subset in the CD133$^+$ fraction. Caffeine treatment caused an earlier increase of BrdU-incorporating cells in both the CD133$^-$ and the CD133$^+$ fraction. Of note, the amount of BrdU-incorporating CD133$^+$ cells increased to 87% at 96 hours, suggesting a caffeine-induced activation of a formerly quiescent CD133$^+$ subset (Fig. 4A, 4B).

In a time-lapse analysis using AnnexinV as marker of early apoptosis, we observed an initial rapid decline of CD133$^+$ cell content after caffeine treatment, followed by a subsequent slower decline (Fig. 4C). Consistently, the fraction of CD133$^+$ AnnexinV$^+$ cells was rather high (up to 8%) during the initial 72 hours, although a smaller yet sustained fraction of CD133$^+$ AnnexinV$^+$ cells was observed up to 168 hours, presumably representing the quiescent CSC fraction progressively recruited to enter an active cell cycle. As apoptosis can be initiated via either the intrinsic (involving activation of caspase 9) or the extrinsic pathway (cleavage of caspase 8) [17], DLD1 cells were next preincubated using either pancaspase-, caspase 8-, or caspase 9-inhibitors before caffeine treatment. Incubation with the pan-caspase inhibitor strongly decreased the fraction of CD133$^+$ AnnexinV$^+$ cells after subsequent caffeine treatment. However, inhibition of either caspase 8 or caspase 9 alone was also sufficient to decrease apoptosis (Fig. 4D), suggesting that caffeine initiated apoptosis through cleavage of caspase 8, but that the recruitment of caspase 9 by a mitochondrial amplification loop was

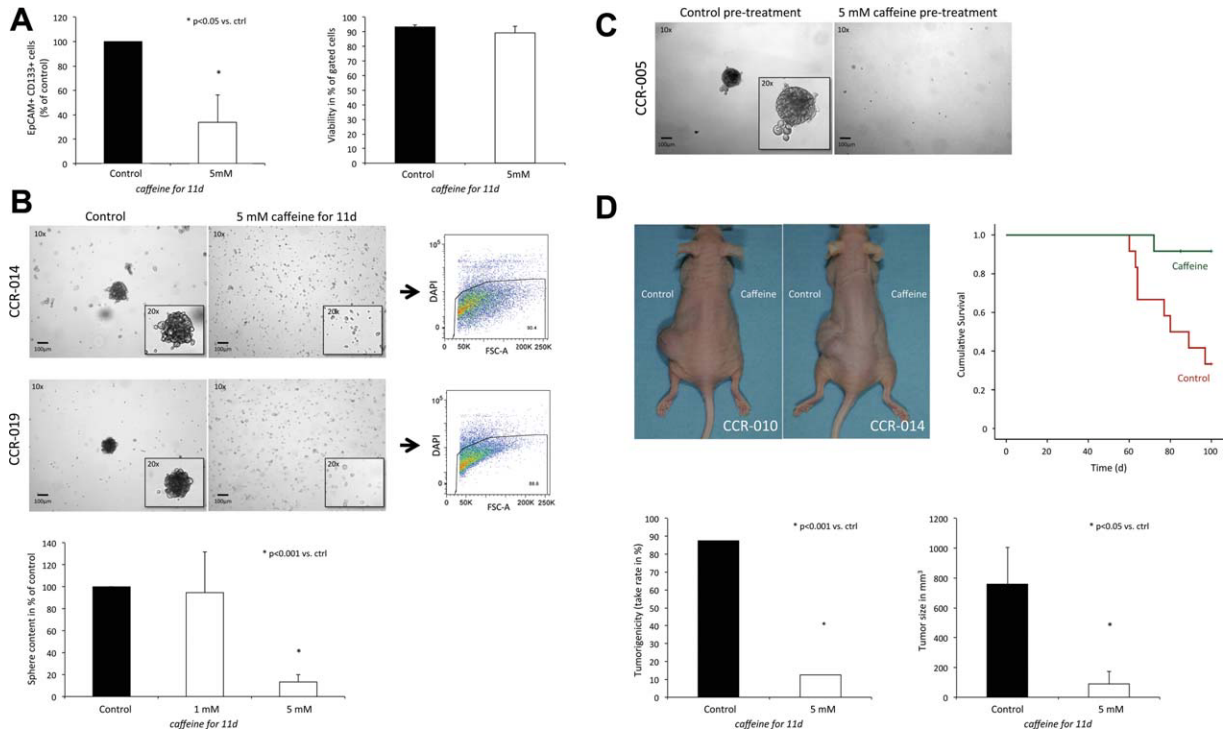

Figure 3. Effects of caffeine on the CD133$^+$ fraction of xenograft-derived primary colon cancer cells. Dissociated single cancer cells, derived from xenograft-expanded surgical colon cancer specimens from five patients, treated for 11 days with either control or caffeine at the indicated concentrations. **(A):** Quantification of the EpCAM$^+$ CD133$^+$ cell fraction (left panel) and viability of the gated cells (right panel; $n = 4$). **(B):** Representative pictures of sphere formation assays (upper left four panels) and assessment of cell viability after caffeine treatment (upper right two panels) for CCR-014 and CCR-019 primary tumor cells. Statistical evaluation is provided for all five tumors ($n = 3$ experiments for 1 mM caffeine, $n = 10$ experiments for 5 mM caffeine; lower panel). **(C):** Caffeine pretreated primary cells, seeded into medium without caffeine. Representative pictures of sphere reformation after 9 days. **(D):** Caffeine- or control-treated primary tumor cells, implanted s.c. into nude mice ($n = 7$ for CCR-010, $n = 5$ for CCR-014, $n = 4$ for CCR-005). Representative pictures of tumor-bearing mice (upper left two panels); Kaplan-Meier curve depicting cumulative event-free long-term survival (event = tumor growth exceeding 1 cm^3; upper right panel). Statistical evaluation is provided for tumorigenicity (lower left panel) and tumor size (lower right panel). Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; EpCAM, epithelial cell adhesion molecule; FSC-A, forward scatter - area.

additionally required for the activation of effector caspases in the CSC fraction.

Caffeine-Induced Depletion of CD133$^+$ Colon Cancer Cells Is Mediated by ATR

Caffeine acts as an unspecific inhibitor of the checkpoint-modulating PIK kinases ataxia telangiectasia mutated (ATM), ATR, and mammalian target of rapamycin (MTOR), without being a global inhibitor of protein kinase activities [18]. To identify the signaling cascade mediating the caffeine-induced depletion of tumorigenic CD133$^+$ cells, we used KU-55399 for ATM inhibition [19] and RAD001 for MTOR inhibition [20]. Because of the lack of ATR-inhibitors and as CHK1 represents the major effector kinase of ATR, we additionally applied the potent, but in comparison with the highly specific ATM inhibitors, less-specific CHK1 inhibitor UCN-01 and its analog SB218078 [21–25]. UCN-01 was chosen as it currently represents the most clinically advanced CHK1 inhibitor, which has already undergone clinical trials, whereas SB218078 was chosen due to its better specificity as it exerts much less inhibitory activity against protein kinase C as compared with UCN-01 [26]. Although treatment with either RAD001 or KU-55399 had no significant effects, UCN-01 and SB218078 both caused a significant decrease of CD133$^+$ cells, suggesting that inhibition of either CHK1 or the upstream PIK kinase ATR mediated the caffeine-induced effects (Fig. 4E).

DLD1 Cells Harboring an Inactivating ATR Mutation Lack the Tumorigenic CD133$^+$ Cell Fraction

To further support that disruption of ATR function was responsible for the caffeine-induced effects, we applied a genetic knock-in model. The hypomorphic *ATR* splice-site "Seckel" mutation $2101^{A\rightarrow G}$ leads to a subtotal depletion of ATR protein but has no gross effects on cancer cell growth or viability [14, 27]. Therefore, parental DLD1 ($ATR^{+/+}$) cells, constitutively expressing ATR protein, were compared with cells homozygously harboring the Seckel mutation ($ATR^{s/s}$ cells) [14], which express no detectable ATR protein (Fig. 5A). $ATR^{+/+}$ and $ATR^{s/s}$ cells were long-term passaged for at least 3 months before analysis of CD133 expression status. Although $ATR^{+/+}$ cells displayed a CD133$^+$ cell population ranging from 3% to 8% (Fig. 5B) when assessed at different time points during cell culture, $ATR^{s/s}$ cells exhibited a near absent CD133$^+$ cell fraction. Consistently, $ATR^{s/s}$ cells were severely impaired in sphere formation capability (Fig. 5C) and completely unable to form tumors in nude mice (take rate 0%; $n = 10$; Fig. 5D, 5E).

Depletion of the Tumorigenic CD133$^+$ Cell Fraction on Continuous siRNA-Mediated ATR-Knockdown

To exclude potential artifacts that might occur due to clonal variability in the *ATR* knock-in experiments, we additionally assessed the effects of continuous ATR protein-depletion on

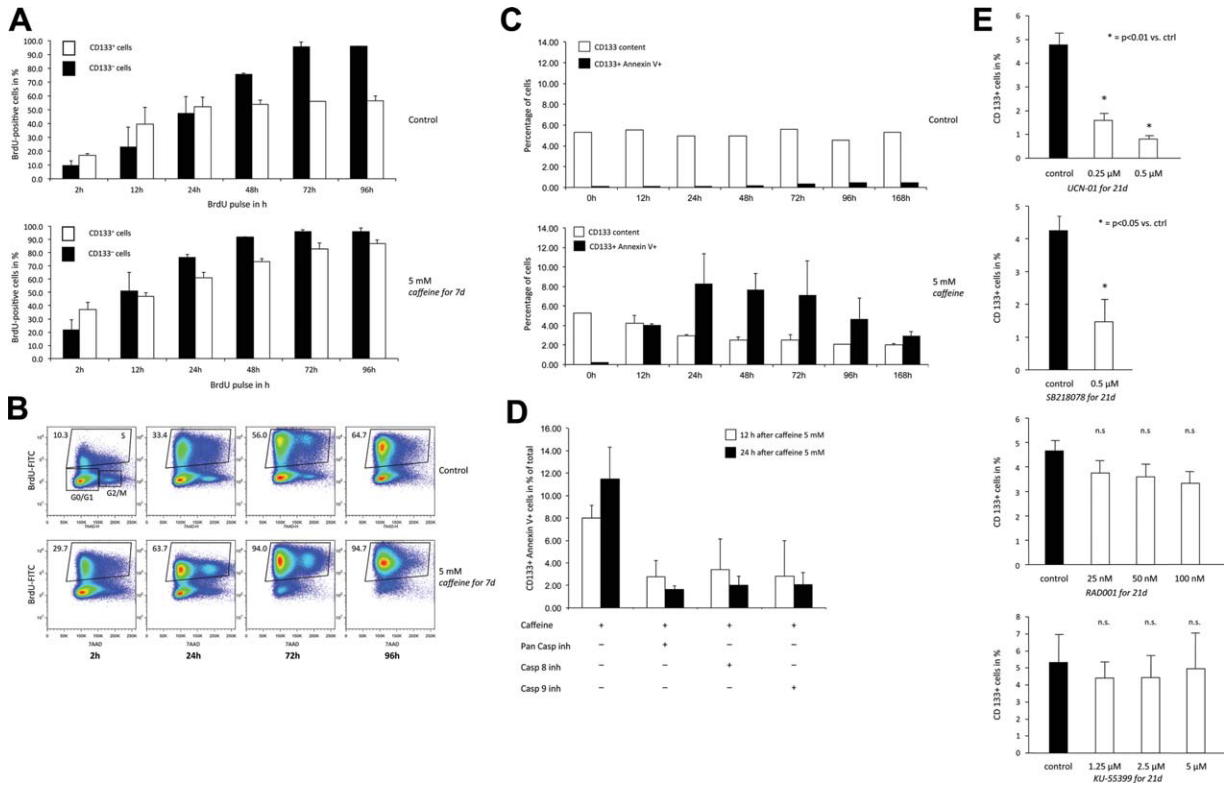

Figure 4. Mechanism of the caffeine-induced depletion of the CD133$^+$ cell fraction. **(A):** BrdU-incorporating fraction of CD133$^+$ DLD1 cells after treatment for 7 days with control (upper panel) or caffeine (lower panel) at 2–96 hours after treatment. **(B):** Representative flow cytometric assessments of cell cycle profiles of CD133$^+$ DLD1 cells after treatment for 7 days with control or caffeine at 2–96 hours after treatment. **(C):** CD133$^+$ fraction and CD133$^+$ Annexin V$^+$ subset of DLD1 cells, either treated with control (upper panel) or caffeine (lower panel), assessed at the indicated time points. **(D):** CD133$^+$ Annexin V$^+$ fraction of caffeine-treated DLD1 cells pretreated with the indicated caspase inhibitors for 2 hours. **(E):** Statistical evaluation ($n = 3$) of the CD133$^+$ fraction of DLD1 cells treated with the indicated PIK kinase or checkpoint inhibitors, respectively, at the indicated concentrations for 21 days. Abbreviations: 7AAD, 7-aminoactinomycin D; BrdU, 5-bromo-2-deoxyuridine; FITC, fluorescein isothiocyanate.

unselected cancer cells, using repetitive applications of ATR-siRNA over a time period of 384 hours. ATR protein-depletion efficiency of >80% was confirmed for all time points starting from 96 hours up to 384 hours (Fig. 6A). ATR siRNA-treated cells displayed a time-dependent reduction of the CD133$^+$ cell fraction when compared with mock-transfected or control siRNA-transfected cells, starting at 96 hours after transfection (Fig. 6B). After 384 hours of repetitive ATR-siRNA treatment, the cells were implanted under the renal capsule of nude mice, and tumor take rate was assessed 30 days later (Fig. 6C). The ATR siRNA-treated cells displayed a reduced but not completely absent capability to form tumors (take rate 33%; $n = 6$) as compared with control cells (Fig. 6D). Two mice bore clearly diminutive tumors, approximating 2 mm^3 in both cases, as compared with 970 mm^3 on average observed in control mice. In these diminutive tumors, we detected only sparse proliferation activity and rare presence of CD133$^+$ cells (<1 cell per high-power field for ATR-siRNA-treated cells vs. 6.1 ± 2.7 for control cells; Fig. 6E).

No Significant Depletion of the Tumorigenic CD133$^+$ Cell Fraction on Continuous siRNA-Mediated CHK1-Knockdown

Analogous to the above experiments using ATR siRNA, a similar set of experiments was performed for ATR's major effector kinase CHK1, using repetitive applications of CHK1 siRNA. In contrast to ATR siRNA-treated cells, which displayed a strong reduction of the CD133$^+$ cell fraction as soon as 96 hours after transfection, CHK1 siRNA-treated cells, even though efficiently depleted of CHK1 protein, did not display a significant reduction of the CD133$^+$ cell fraction over a time period of 264 hours when compared with control cells (data not shown).

Depletion of CD133$^+$ Colon Cancer Cells on ATR-Inhibition Is Fanconi Anemia Pathway-Independent

As ATR-inhibition causes disruption of the Fanconi anemia (FA) DNA-repair pathway [28], we tested whether this pathway contributed to the ATR inhibition-mediated effects on CD133$^+$ cells. The CD133$^+$ cell fraction of RKO colon cancer cells was compared with the CD133$^+$ fraction of RKO cells engineered to harbor inactivating deletions of either the *FANCC* or *FANCG* gene [13]. No significant differences were observed in this FA model, excluding the FA pathway as a major contributing factor to the ATR inhibition-induced depletion of CD133$^+$ cells (data not shown).

Induction of Stalled Replication Forks Increases the Effects of ATR/CHK1-Inhibition on the CD133$^+$ Cell Fraction

ATR acts as a central regulator of the replication checkpoint and participates in the detection and repair of endogenous and exogenously induced stalled replication forks (SRF) via

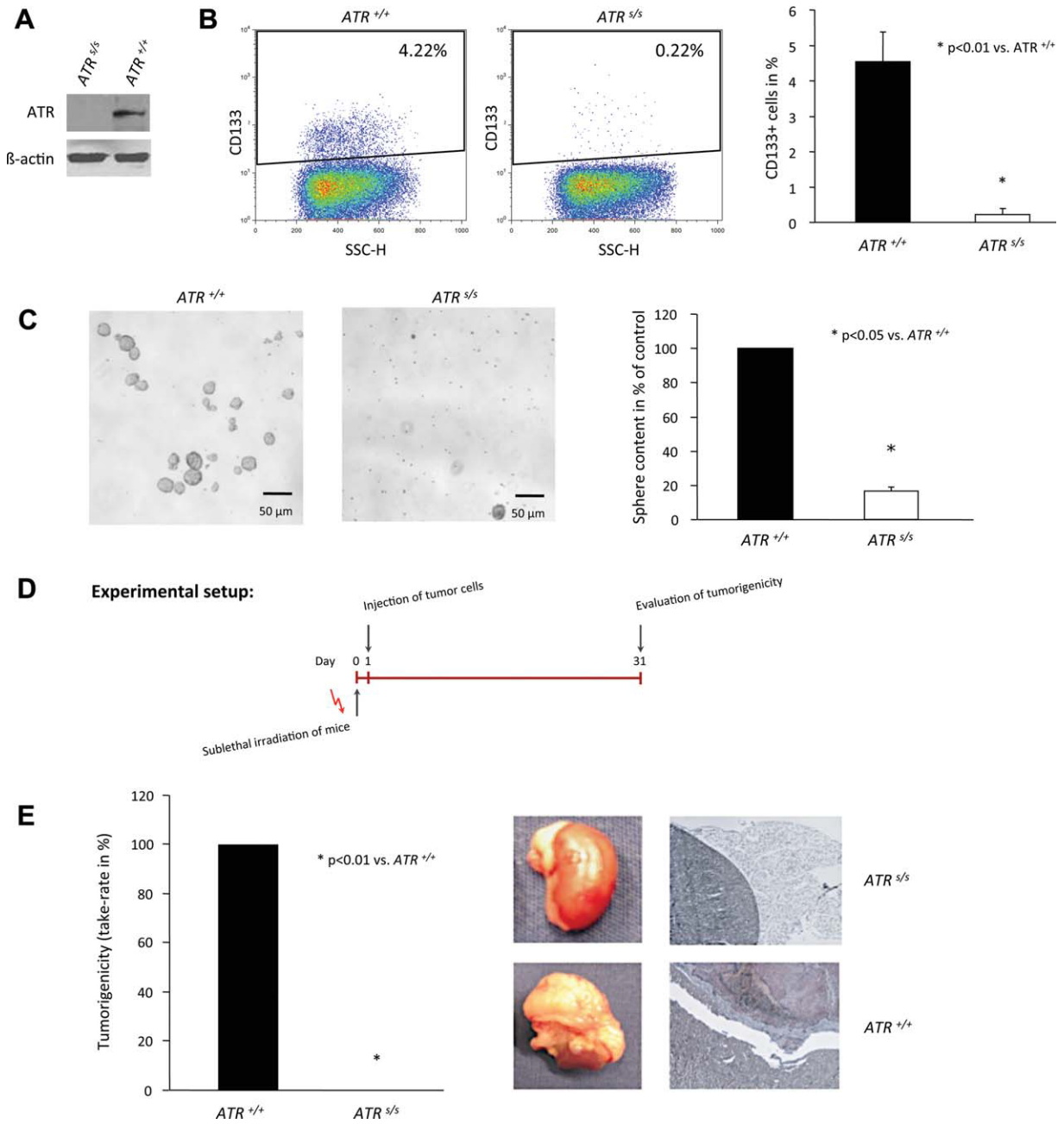

Figure 5. Reduced tumorigenicity of colon cancer cells harboring an inactivating *ATR* mutation. **(A):** ATR protein content of $ATR^{+/+}$ and $ATR^{s/s}$ cells, as assessed by Western blotting. **(B):** $CD133^{+}$ cell fraction of $ATR^{+/+}$ and $ATR^{s/s}$ cells: representative results (left two panels) and statistical analysis ($n = 5$, assessed at five time points during culture, right panel). **(C):** Sphere formation capacity of $ATR^{+/+}$ cells and $ATR^{s/s}$ cells: representative results (left two panels) and statistical evaluation ($n = 3$, right panel). **(D):** Experimental setup for the in vivo experiments. **(E):** In vivo tumorigenicity, evaluated 30 days after injection of $ATR^{+/+}$ ($n = 10$) or $ATR^{s/s}$ ($n = 10$) cells under the renal capsule of nude mice: statistical evaluation (left panel) and representative macroscopic and microscopic pictures (right panel). Abbreviations: ATR, ataxia telangiectasia and Rad3 related; SSC-H, side scatter - height.

phosphorylation of the checkpoint kinase CHK1 [29]. Consistent with previous reports describing amplified checkpoint activation in some CSC [11, 12], upregulation of CHK1 phosphorylation was significantly more pronounced in the $CD133^{+}$ than in the $CD133^{-}$ cell fraction on treatment with the SRF-inducing ICL-agent mitomycin C, when compared with the respective untreated cell fractions. Importantly, the increased CHK1 phosphorylation levels in the $CD133^{+}$ cell fraction were not merely ascribable to increased levels of total CHK1 protein (Fig. 7A, left panel). As ICL-inducing platinum compounds are more commonly used than mitomycin C for the treatment of colorectal cancer in the clinical setting, the above results were additionally validated using cisplatin, a classic platinum ICL-agent, yielding similar results (Fig. 7A, right panel).

To test whether consequently, ICL-agents would synergistically add to the preferential depletion of $CD133^{+}$ cells through inhibition of the ATR/CHK1 axis, DLD1 cells were treated with caffeine, the CHK1-inhibitor SB218078 or the ICL-agent cisplatin alone, or using different combinations of

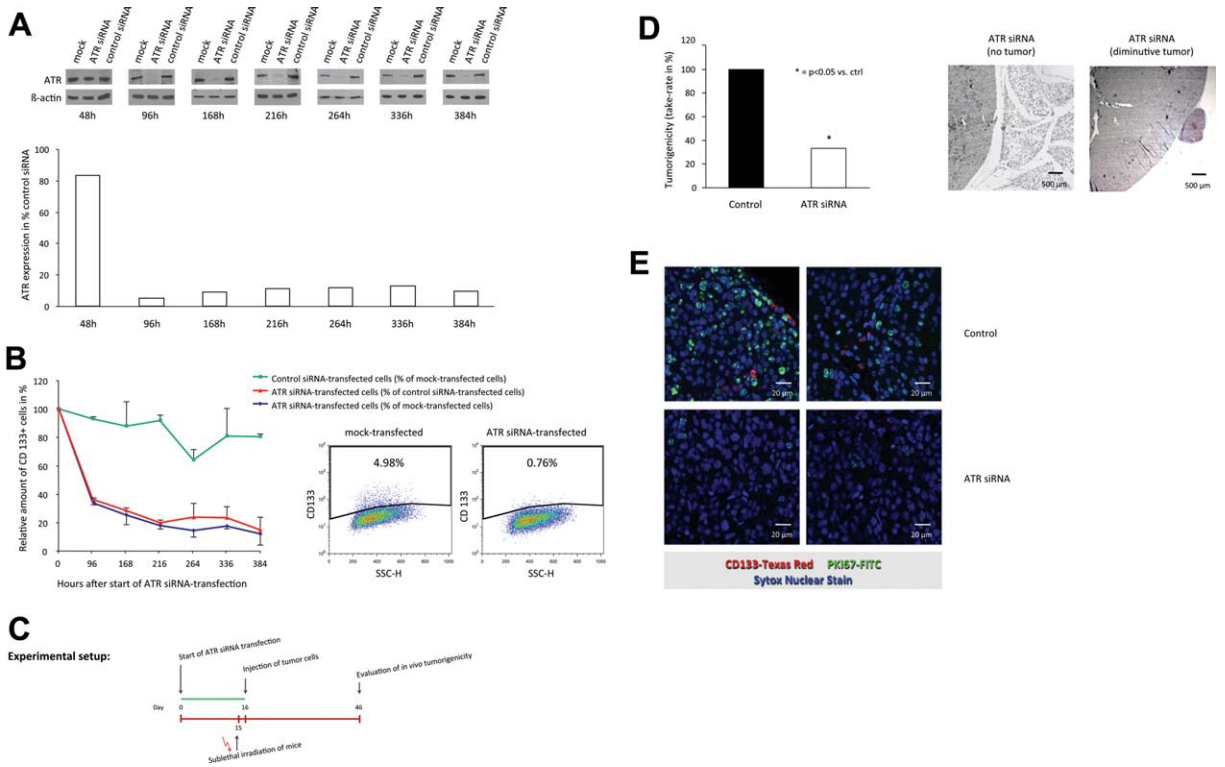

Figure 6. Decreased CD133$^+$ fraction and reduced tumorigenicity of colon cancer cells on ATR protein-depletion: **(A):** Representative results from one of two experiments, showing ATR protein content of mock-, ATR siRNA-, and control siRNA-transfected DLD1 cells at the indicated time points, as assessed by Western blotting (upper panel), and corresponding densitometric quantification (lower panel). **(B):** Flow cytometric assessment of the relative CD133$^+$ content of mock-, ATR siRNA-, and control siRNA-transfected DLD1 cells, compared with each other at the indicated time points (left panel). CD133$^+$ fraction of mock- or ATR siRNA-transfected cells after 384 hours of repetitive siRNA application (right two panels). **(C):** Experimental setup for the in vivo experiments. **(D):** In vivo tumorigenicity, evaluated 30 days after injection of ATR siRNA-transfected cells: statistical evaluation ($n = 6$, left panel) and representative microscopic pictures showing no or diminutive tumor growth, respectively (right two panels). **(E):** Histological analysis for the presence of CD133$^+$cells (red) in tumors from control- or ATR siRNA-transfected cells. Cell proliferation was assessed by Ki67 staining (green). Nuclei were identified by Sytox Blue staining (blue). Abbreviations: ATR, ataxia telangiectasia and Rad3 related; FITC, fluorescein isothiocyanate; SSC-H, side scatter - height.

these agents. Caffeine caused a significant decrease of CD133$^+$ cells as early as 5 days after initiation of treatment and a nearly complete elimination on longer treatment (21 days). Treatment with the CHK1-inhibitor SB218078 at 10 or 20 nM resulted in a significant decrease of CD133$^+$ cells only after 21 days (Fig. 7B). Consistent results were obtained when using Colo320 cells (Fig. 7C, left panel) and further supported by the observation that the CD133$^+$ AnnexinV$^+$ cell fraction inversely correlated with the total CD133$^+$ cell fraction (Fig. 7C, right panel). In contrast, we observed no decrease of CD133$^+$ cells on treatment with cisplatin alone at 6.6 μM for 2 days (Fig. 7D) in either DLD1 or Colo320, whereas pretreatment with either caffeine or SB218078 followed by treatment with cisplatin strongly reduced the CD133$^+$ fraction of both cell lines already after short-term treatment (Fig. 7E).

Discussion

We demonstrate here that inhibition of ATR function depletes the tumorigenic CD133$^+$ fraction of established colon cancer cell lines as well as xenograft-derived primary colon cancer cells. This effect translated into a markedly reduced tumorigenicity of the remaining cells, as shown by an impaired sphere formation capacity in vitro [5, 7] as well as a strongly reduced capability to form tumors in vivo. Consistent with previous reports describing amplified checkpoint activation and increased DNA repair to be distinct features of some CSC [11, 12], CD133$^+$ cells displayed a stronger activation of the ATR-dependent DDR on treatment with ICL-agents than did CD133$^-$ cells, as evidenced by a more pronounced increase in phosphorylation of ATR's major effector kinase CHK1. Importantly, the depletion of CD133$^+$ cells was enhanced on subsequent treatment with ICL-agents, suggesting that inhibition of ATR might reverse the chemoresistance of CSC toward ICL-agents in the clinical setting and could thus serve as a novel therapeutic strategy for patients suffering from colon cancer.

As the PIK kinases ATM, ATR, MTOR, and DNA-PK all play pivotal roles in cell cycle checkpoint functions and all except DNA-PK are effectively inhibited by caffeine [18], caffeine was used as a screening approach to modulate checkpoint function in colon cancer cells. Caffeine treatment virtually abolished the CD133$^+$ cell fraction and was accompanied by a decreased in vitro and in vivo tumorigenicity of the remaining cell population, providing functional evidence for a successful targeting of the tumor-initiating CSC fraction. Depletion of CD133$^+$ cells was observable as early as 5 days after caffeine administration and further enhanced after longer exposure, indicating that prolonged treatment was required for the complete exhaustion of the CD133$^+$ subpopulation, first through the elimination of the rapid cycling cell fraction and consecutively through activation of a slow cycling or even

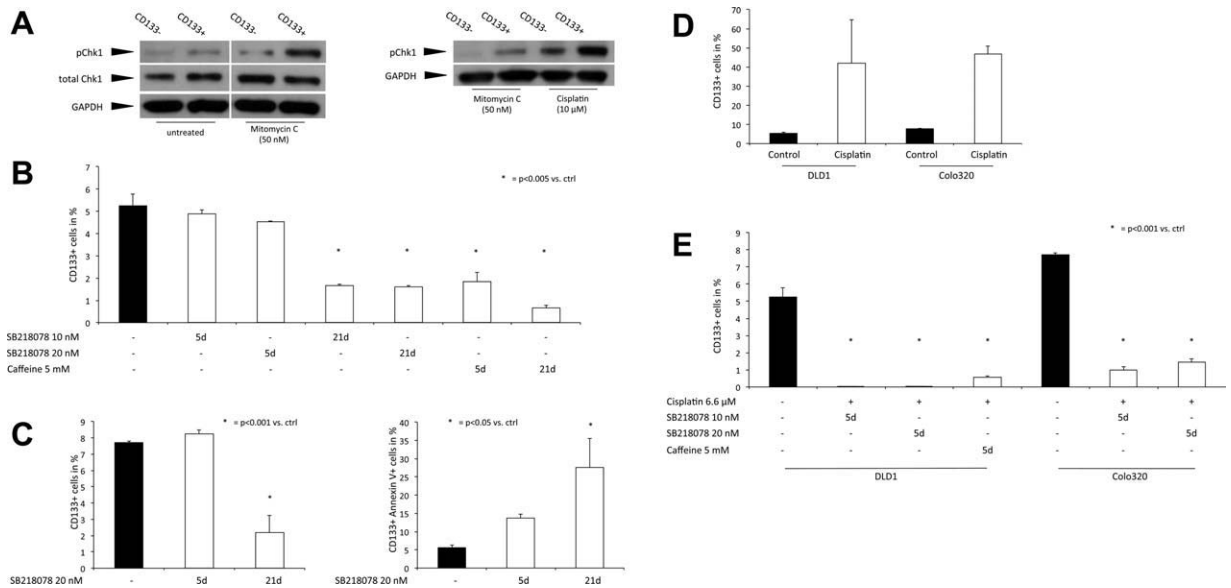

Figure 7. Additive effects of interstrand-crosslinking (ICL)-agents on the depletion of $CD133^+$ cells after inhibition of the ataxia telangiectasia and Rad3 related (ATR)/checkpoint homolog (CHK1) axis. **(A):** Western blotting showing increased Chk1 phosphorylation in $CD133^+$ as compared with $CD133^-$ DLD1 cells on treatment with ICL-agents: Comparison of pCHK1 and CHK1 protein levels in $CD133^+$ and $CD133^-$ cells either left untreated or treated with mitomycin C (left panel), representative results from $n = 2$ experiments are shown. Comparison of pCHK1 protein levels in $CD133^+$ and $CD133^-$ cells treated with either mitomycin C or cisplatin (right panel), representative results from $n = 3$ experiments are shown. **(B):** $CD133^+$ fraction of DLD1 cells after treatment with SB218078 or caffeine for 5 or 21 days, respectively. For illustrative purposes, data for long-term SB218078 treatment were taken from Figure 4E, for caffeine treatment at 5 mM from Figure 2B. **(C):** Total $CD133^+$ cell fraction (left panel) and $CD133^+$ Annexin V^+ subset (right panel) of Colo320 cells after treatment with SB218078 for 5 or 21 days (left panel). **(D):** $CD133^+$ fraction of DLD1 and Colo320 cells after treatment with cisplatin at 6.6 μM for 5 days. **(E):** $CD133^+$ fraction of DLD1 and Colo320 cells after pretreatment with SB218078 or caffeine for 5 days, followed by cisplatin for 2 days. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

quiescent fraction. Consistently, caffeine increased the proliferating fraction of $CD133^+$ cells in our experiments.

A panel of small molecule inhibitors was applied to dissect the contributions of the different PIK kinases on the caffeine-induced preferential depletion of $CD133^+$ cells. As no specific ATR-inhibitors are currently available, several potent, but in comparison with the highly specific ATM inhibitors, less-specific inhibitors of CHK1 as the major effector kinase of ATR were used as surrogates for ATR inhibition. In contrast to the ATM inhibitor KU-55399 and the MTOR inhibitor RAD001, only CHK1 inhibitors mimicked the effects of caffeine. As CHK1 activity itself is only marginally suppressed by caffeine [18], whereas the upstream PIK kinase ATR is potently inhibited, these data suggested that the caffeine-induced depletion of $CD133^+$ cells was mediated through direct inhibition of ATR followed by indirect inhibition of its main effector kinase CHK1. Importantly, caffeine exerted its detrimental effects on $CD133^+$ cells already after short-term treatment, whereas the effects of CHK1 inhibitors were observable only after long-term treatment. To exclude different pharmacokinetic properties of the used agents as the underlying reason for the observed differences, our findings were corroborated by a set of siRNA experiments. Consistently, knockdown of CHK1 protein expression over 264 hours did not lead to comparable detrimental effects on the $CD133^+$ cell population as did ATR protein depletion. Furthermore, we analyzed an isogenic FA knockout model [13, 30], as ATR had been linked to the FA DNA-repair pathway [28], but found no evidence for an impact of FA pathway abrogation on the depletion of $CD133^+$ colon cancer cells. Together, these data suggest that besides CHK1 as the major effector kinase of ATR, other ATR-dependent, but FA-independent pathways are operative in this setting.

The complete disruption of the *ATR* gene is a lethal event in human somatic cells [31] and no applicable cellular model presently exists to investigate the null state of the *ATR* gene. However, the hypomorphic *ATR*-inactivating splicing mutation $2101^{A\rightarrow G}$, naturally found in Seckel syndrome patients [32], causes subtotal depletion of ATR protein without gross effects on cancer cell growth or viability [14, 30]. Therefore, cancer cells homozygously harboring this mutation ($ATR^{s/s}$ cells) were used as a highly specific tool to model ATR inhibition in tumors. $ATR^{s/s}$ cells were virtually depleted of $CD133^+$ cells as compared with parental $ATR^{+/+}$ cells. Consistently, $ATR^{s/s}$ cells were impaired in sphere formation capacity and unable to form tumors in vivo. $ATR^{s/s}$ cells did not show significant differences in proliferation rates as compared with their $ATR^{+/+}$ counterparts, excluding that their loss of tumorigenicity was attributable to a hypothetical cell cycle arrest. It should be noted that a limitation of our genetic *ATR* model is that confounding artifacts due to clonal variability cannot definitively be excluded [30]. Therefore, our data require cautious interpretation, especially when considering the CD133 expression status of the originally derived $ATR^{s/s}$ cell clones. As can be derived from our initial experiments, tumorigenicity was mainly restricted to the $CD133^+$ cell fraction of DLD1 colon cancer cells, which constituted only about 5% of the unselected DLD1 cell population. Thus, the engineered $ATR^{s/s}$ cells were more likely originally derived from a $CD133^-$ cell clone, which according to our data, would be expected not to be capable of regenerating tumorigenic $CD133^+$ cells, at least in our short-term experimental setting (14 days). On the other hand, it remains a controversial issue whether non-CSC or a subpopulation of them might be able to regenerate CSC in the long run, or correspondingly, whether $CD133^-$ $ATR^{+/+}$ cells might at some point regenerate $CD133^+$ cells [33]. Taken together,

our data demonstrate that ATR-deficient $CD133^-$ cancer cells retain a nontumorigenic phenotype for at least several months during cell culture.

To exclude potential artifacts due to clonal variability, we employed a third model of ATR function, using RNA-interference through repetitive application of ATR siRNA, which facilitated the continuous depletion of ATR protein in unselected colon cancer cell populations. Similar to the results obtained in the genetic model, we observed a time-dependent decrease of the $CD133^+$ cell fraction in ATR siRNA-treated cells along with a concomitant reduction of the in vivo tumorigenicity of the remaining cell population, strongly indicative of a successful targeting of the tumor-initiating stem cell fraction. In contrast to $ATR^{s/s}$ or caffeine-treated cells, however, ATR siRNA-treated cells did not exhibit a complete abrogation of in vivo tumor formation in all animals. This could most likely be ascribed to an inevitable methodological shortcoming of experiments applying siRNA technology, that is, the incomplete targeting on the cellular level, generally leaving a remaining subpopulation of not efficiently siRNA-transfectable cells (including $CD133^+$ cells), unaffected. Accordingly, traceable amounts of ATR protein were still detectable in the ATR siRNA-treated cell population after 384 hours of repetitive siRNA-application. As a consequence, the decreased, but maybe not absent, tumorigenic cell fraction on ATR siRNA treatment would be expected to lead to a significantly decreased, but not absent, tumor take rate, as observed in our experiments. Interestingly, in those rare instances, in which tumors were generated by ATR siRNA-treated cell populations, these tumors were clearly diminutive as compared with those observed in control mice. This could be explained by the decreased fraction of tumorigenic $CD133^+$ cells in the ATR siRNA-treated population, as a smaller fraction of tumor-initiating cells could perceivably also account for a decreased tumor size in those rare instances of successful tumor formation. Indeed, a very rare occurrence of $CD133^+$ cells was observable in tumors that originated from ATR siRNA-treated cells. Another explanation, consistent with the observed sparse proliferation activity of the diminutive tumors in vivo, would be that these tumors did not arise from the successfully eliminated tumor-initiating cell fraction but rather from an untargeted subset of short-lived transient amplifying cells, which only divide a finite number of times until they become terminally differentiated and finally undergo senescence [34].

A preferential activation of the DDR, comprising both amplified checkpoint activation and increased DNA-repair, has previously been proposed as a likely mechanism of CSC drug-resistance [11, 12] and could also explain the increased sensitivity of $CD133^+$ colon cancer cells toward ATR inhibition; ATR is a central regulator of the replication checkpoint, which blocks cell cycle progression on detection of endogenous or exogenously induced SRF. In this process, ATR stabilizes SRF via its main effector kinase CHK1 and prevents the inappropriate processing of DNA [29]. Accordingly, cells harboring a complete disruption of the *ATR* gene display increased chromosome breaks even in the absence of exogenous replication stress, most likely induced through SRF occurring during normal cellular proliferation, and are not viable over extended periods of time [31]. The significantly stronger upregulation of CHK1 phosphorylation in the $CD133^+$ as compared with the $CD133^-$ cell fraction on treatment with SRF-inducing ICL-agents in our experiments thus supports a preferential activation of the ATR-dependent DDR also in colon CSC.

Consistently, treatment with SRF-inducing ICL-agents accelerated the depletion of $CD133^+$ cells on ATR inhibition, further supporting that the detrimental effects of caffeine specifically on $CD133^+$ cells were attributable to the particularly reduced capability of these cells to repair, endogenously or exogenously inflicted, SRF when ATR function was impaired. It is tempting to speculate that the impaired DNA repair capability of $CD133^+$ cells in response to ATR inhibition could be ascribed structurally to differences in chromatin compaction between $CD133^+$ and $CD133^-$ cells. Overall, chromatin accessibility, a dynamic process largely mediated by chromatin compaction, represents an innate property of stem cells, which is lost during differentiation [35]. The degree of chromatin compaction, on the other hand, determines at least in part the extent of DNA damage, the feasibility of DNA repair [36], and the strength of the DDR [37] and could thus explain a particularly strong dependence of CSC on an intact DDR.

On confrontation with DNA damage, the DDR mediates whether cells undergo a replication arrest to allow DNA repair, bypass the DNA damage and continue to replicate DNA, or eventually, undergo apoptosis [38]. We found that after caffeine treatment, apoptotic cell death did not occur immediately in $CD133^+$ cells, but progressively increased with cumulative BrdU incorporation, excluding cytotoxicity as the sole source of the caffeine-induced effects. Notably, a small fraction of $AnnexinV^+$ $CD133^+$ cells was detectable up to 168 hours after treatment initiation, likely representing the CSC fraction progressively recruited to enter an active cell cycle. Consistently, after an initial caffeine-induced increase of $CD133^+$ $AnnexinV^+$ cells, their amount subsequently declined, paralleling the decline of total $CD133^+$ cell numbers. Thus, the caffeine-induced depletion of $CD133^+$ cells was at least in part attributable to proliferation-dependent induction of apoptosis. As apoptosis was triggered by caspase 8 and reinforced by a mitochondrial amplification loop involving the recruitment of caspase 9, sensitization to extrinsic receptor-mediated apoptosis might provide another tool for the specific depletion of the CSC fraction in colon cancer.

Conclusion

Using three independent model systems, that is, pharmacological ATR inhibition, genetic inactivation of the *ATR* gene, and RNA interference-mediated ATR protein depletion, we found that inhibition of ATR function depleted the tumorigenic $CD133^+$ cell fraction of established human colon cancer cell lines as well as xenograft-derived primary colon cancer cells. This effect was attributable at least in part to apoptosis, accelerated on cotreatment with common chemotherapeutics that generate SRF, and accompanied by a drastically decreased in vitro and in vivo tumorigenicity of the remaining cells. Mechanistically, the preferential depletion of tumorigenic $CD133^+$ cells was attributable to the preferential activation of the ATR-dependent DDR in these cells. Our study thus illustrates a novel approach to selectively eliminate the tumorigenic cell population in colon cancer. As the caffeine blood levels required for inhibiting ATR function cannot be achieved in vivo due to the narrow therapeutic window and the pronounced cardiovascular side effects of caffeine and its derivatives, our study provides a strong rationale for the pharmaceutical development of specific ATR inhibitors as a potentially powerful approach to eliminate CSC in colorectal cancer [39–41].

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Nodal/Activin signaling drives self-renewal and tumourigenicity of pancreatic cancer stem cells and provides a target for combined drug therapy.

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We have provided conclusive evidence for the re-activation of the developmental Nodal/Activin pathway in pancreatic cancer stem cells, which strongly increases their plasticity and aggressiveness. Intriguingly, this pathway is not only active in pancreatic cancer stem cells, but also in pancreatic stellate cells (representing a putative CSC niche). Furthermore, we demonstrated that the Nodal/Activin pathway is essential for the self-renewal capacity and the stemness properties of pancreatic CSC, and therefore represents a novel therapeutic target. In this context we have shown that targeting of the Nodal/Activin pathway but not of TGF-β by small molecule inhibitors or genetic knockdown, respectively, eliminates cancer stem cells and thus the tumourigenic potential of pancreatic cancer cells. Embarking on further preclinical studies we have shown that the cancer stem cell compartment can be severely altered by inhibition of this pathway, resulting in chemo-sensitization of the cancer stem cells, which then can be eliminated by standard chemotherapy resulting in disease stabilization. However, a major challenge in pancreatic cancer remains drug delivery, since poor vascularization and massive stroma content are hallmarks of the disease. Intriguingly, we were able to overcome this hurdle by simultaneous targeting of the sonic hedgehog pathway as a crucial signaling component for pancreatic stellate cells and other stromal cells, an observation that confirms the results of a study that we published previously.(Mueller et al., 2009) The resulting triple therapy containing a Nodal/Activin inhibitor, a hedgehog pathway inhibitor, and a chemotherapeutic agent resulted in long-term survival of all mice in a clinically most relevant primary pancreatic cancer model, while mice receiving only the chemotherapeutic agent had to be sacrificed within a few weeks due to rapid tumour progression.

I contributed to the design of this study, performed the experiments together with the other authors, analyzed and interpreted the results. I also participated in the writing of the manuscript, with input from the rest of the authors and under the supervision of the thesis director Prof. Christopher Heeschen.

Cell Stem Cell
Article

Nodal/Activin Signaling Drives Self-Renewal and Tumorigenicity of Pancreatic Cancer Stem Cells and Provides a Target for Combined Drug Therapy

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SUMMARY

Nodal and Activin belong to the TGF-β superfamily and are important regulators of embryonic stem cell fate. Here we investigated whether Nodal and Activin regulate self-renewal of pancreatic cancer stem cells. Nodal and Activin were hardly detectable in more differentiated pancreatic cancer cells, while cancer stem cells and stroma-derived pancreatic stellate cells markedly overexpressed Nodal and Activin, but not TGF-β. Knockdown or pharmacological inhibition of the Nodal/Activin receptor Alk4/7 in cancer stem cells virtually abrogated their self-renewal capacity and in vivo tumorigenicity, and reversed the resistance of orthotopically engrafted cancer stem cells to gemcitabine. However, engrafted primary human pancreatic cancer tissue with a substantial stroma showed no response due to limited drug delivery. The addition of a stroma-targeting hedgehog pathway inhibitor enhanced delivery of the Nodal/Activin inhibitor and translated into long-term, progression-free survival. Therefore, inhibition of the Alk4/7 pathway, if combined with hedgehog pathway inhibition and gemcitabine, provides a therapeutic strategy for targeting cancer stem cells.

INTRODUCTION

Although pancreatic ductal adenocarcinoma has become the subject of increasing research efforts over the past decades, poor response to therapy with subsequent dismal survival has remained the hallmark of this disease. Recent evidence from our and other laboratories suggests that pancreatic carcinomas harbor a distinct subpopulation of putative cancer stem cells (CSCs) defined by their self-renewal capacity, differentiation ability, exclusive in vivo tumorigenicity (Hermann et al., 2007; Li et al., 2007), and ability to drive metastasis (Hermann et al., 2008). Most importantly, CSCs have also been proposed as the major source of resistance toward conventional chemotherapy and radiotherapy (Bar et al., 2007; Hermann et al., 2007; Mueller et al., 2009). Therefore, novel therapies capable of eliminating CSCs while leaving normal stem cells unaffected are urgently needed.

Members of the TGF-β family, namely Bone Morphogenic Proteins (BMPs), TGF-β, and Nodal/Activin, exert multiple, and sometimes opposing, effects on a variety of cell types depending on the cellular context, including the stage of the disease, the local environment, and the identity and the dosage of the ligand (Massagué, 2008; Watabe and Miyazono, 2009). Nodal and Activin as secreted proteins are expressed during embryonic development and are implicated in developmental events such as mesoderm formation and left-right axis specification. Moreover, they were shown to be essential for human embryonic stem cell (ESC) maintenance (Vallier et al., 2005; Xiao et al., 2006), but their role in cancer still remains poorly defined. Nodal and Activin bind to their common receptors, the *A*ctivin-*like* (Alk) type I receptors Alk4 and 7, while Cripto-1 constitutes an important coreceptor for Nodal signaling only (Strizzi et al., 2005). Recently, Nodal signaling was linked to a more aggressive phenotype in melanoma and breast cancer cells (Topczewska et al., 2006). Furthermore, inhibition of Nodal signaling has been shown to reduce tumorigenicity in melanoma cell lines, suggesting a potential role in tumor-initiating cells (Postovit et al., 2008). Encouraged by these reports, we investigated the role of the Nodal/Activin signaling cascade in the tumorigenic stem cell compartment of

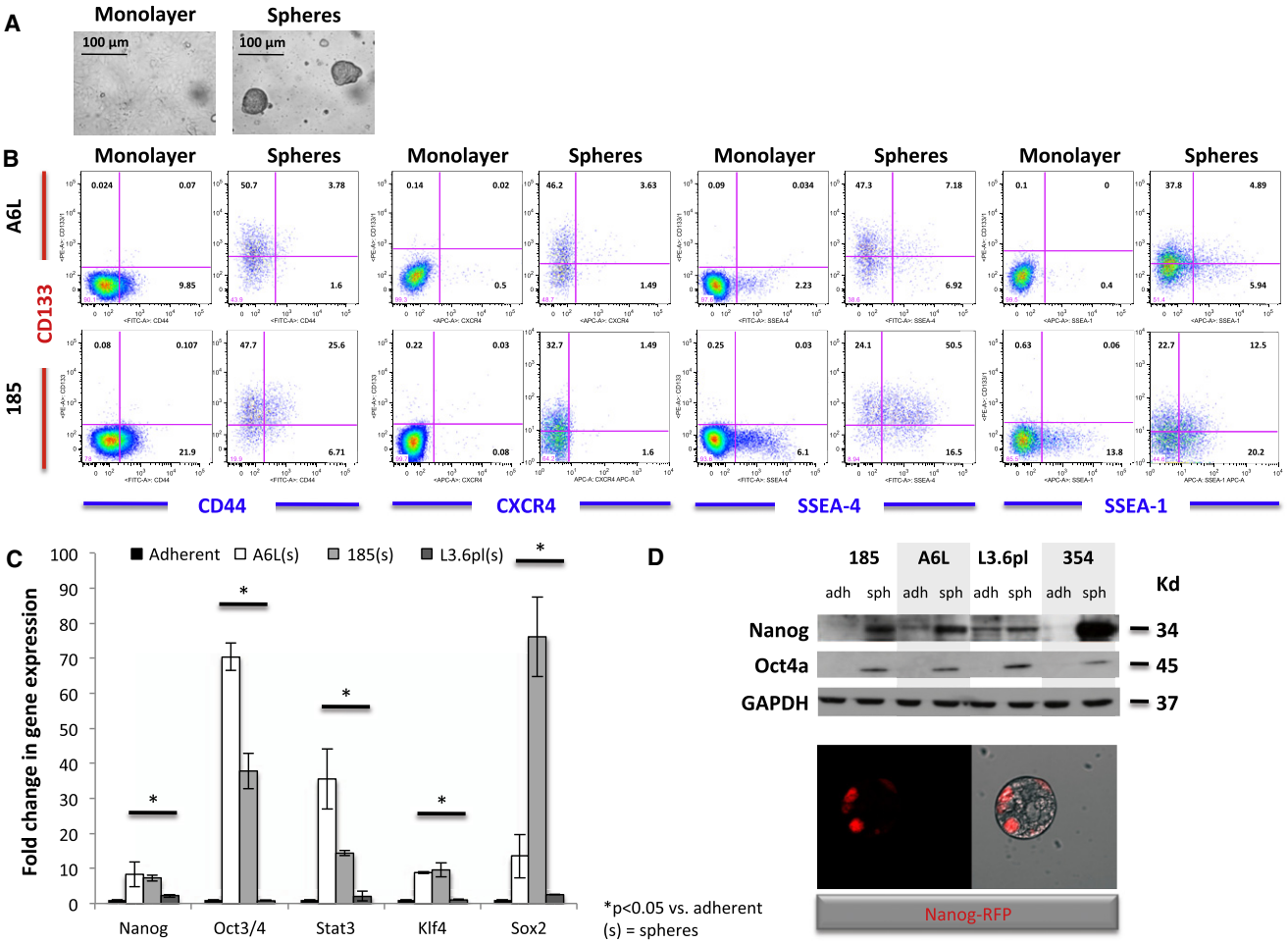


Figure 1. Sphere-Derived Pancreatic CSCs Express Pluripotency Markers

(A) Morphology of pancreatic cancer cells derived from xenografts and freshly isolated human tissue grown as monolayers or spheres.

(B) Flow cytometry analysis for CD44, CD133, CXCR4, SSEA-4, and SSEA-1 as cancer stem cell markers in spheres as compared with adherent cells from A6L or 185 tumors.

(C) qPCR analysis of pluripotency-associated genes in adherent cells versus spheres. Data are normalized to GAPDH expression and are presented as fold change in gene expression relative to adherent cells.

(D) Western blot analysis of Nanog, Oct4a, and GAPDH in spheres as compared with adherent cells. Nanog promoter RFP reporter construct illustrates the presence of single Nanog promoter^{+} cells in spheres.

pancreatic cancer, and its potential as a therapeutic target for the successful elimination of pancreatic CSCs as the root of this deadly disease.

RESULTS

Pancreatic CSCs Express Pluripotency-Associated Markers

We have shown that primary pancreatic CSCs can be enriched in vitro as anchorage-independent spherical colonies termed spheres (Hermann et al., 2007). These spheres are composed of a small number of cells with stem cell-like properties including the ability to form secondary spheres as well as more differentiated progenies. Recently, we also reported the enrichment of pancreatic CSCs within the CD133^{+}-expressing cell population as assessed by flow cytometry (Hermann et al., 2007). Therefore, for the present studies, we used these two supplementary methods for studying pancreatic CSCs.

A total number of eight human pancreatic adenocarcinoma xenografts were used, with A6L, 185, JH051, 247, and 198 being described earlier as primary tumors or tumor-derived primary cell lines (Jones et al., 2008; Rubio-Viqueira et al., 2006), and with 265, 286, and 354 produced by the same technique. Importantly, all cells for in vitro experiments were freshly isolated from early passage xenografts. Isolated cells from these xenografts were cultured as adherent cells (monolayer) or anchorage-independent spheres at low passages (Figure 1A). Moreover, three established pancreatic cancer cell lines (L3.6pl, MiaPaCa2, and Panc1) were used. Cells were phenotyped by flow cytometry for the expression of CD133, CD44, CXCR4, SSEA-4, and SSEA-1. As previously reported, spheres are enriched in CD133^{+} cells, as well as several other markers that have been associated with a CSC phenotype such as CXCR4, SSEA-4, and SSEA-1, as

compared with adherent cells (Hermann et al., 2007; Scaffidi and Misteli, 2011). In contrast, cells expressing adhesion molecules such as CD44 (Figure 1B) and EpCAM (data not shown) were not consistently enriched in sphere culture, mostly likely reflecting anchorage-independent culture conditions, and were therefore not linked to a CSC phenotype in cultured cells (Figure S1A available online).

Next, the expression of pluripotency-associated genes (*Nanog*, *Oct3/4*, *Stat3*, *Klf4*, and *Sox2*) was determined by real-time PCR. Expression of pluripotency-associated genes was significantly higher in first generation sphere culture (d7) versus 70% confluent monolayer culture (Figure 1C). Intriguingly, the expression levels observed for pancreatic spheres were comparable to those of human ESCs (data not shown). Expression of protein levels was validated for Oct4a and Nanog by western blotting and using a Nanog promoter reporter construct (Figure 1D).

Components of the Nodal/Activin Signaling Cascade Are Overexpressed in Primary Pancreatic CSCs

Because the Nodal/Activin pathway is reportedly inactive in adult tissue (Hendrix et al., 2007; Topczewska et al., 2006), we determined whether this pathway is reactivated in pancreatic cancer (stem) cells by assessing mRNA expression for its components, namely *Nodal*, *Cripto-1*, *FoxH1*, *Smad2*, *Smad4*, *Gdf1*, *Activin*, and *Alk4*. Real-time PCR demonstrated that Nodal/Activin signaling-related genes are significantly overexpressed in first-passage spheres as compared with those in adherent cells, although marked differences in mRNA expression between the various tumors can be noted (Figure 2A). Interestingly, the expression further and strongly increased in second-passage spheres (Figure 2B). Western blot analysis demonstrated that Nodal is consistently and strongly overexpressed in spheres as compared with adherent cells on the protein level (Figure 2C), and that Alk4$^+$ as well as Cripto$^+$ cells are also enriched in sphere culture as determined by flow cytometry (Figure S1B). In contrast, mRNA levels for *TGF-β1*, TGF-β type I receptor/*Alk5*, and *TGF-β type II* receptor did not differ between sphere-derived cells and adherent cells, while flow cytometry revealed even decreased numbers of Alk5$^+$ cells in spheres (Figures S1B and S1C).

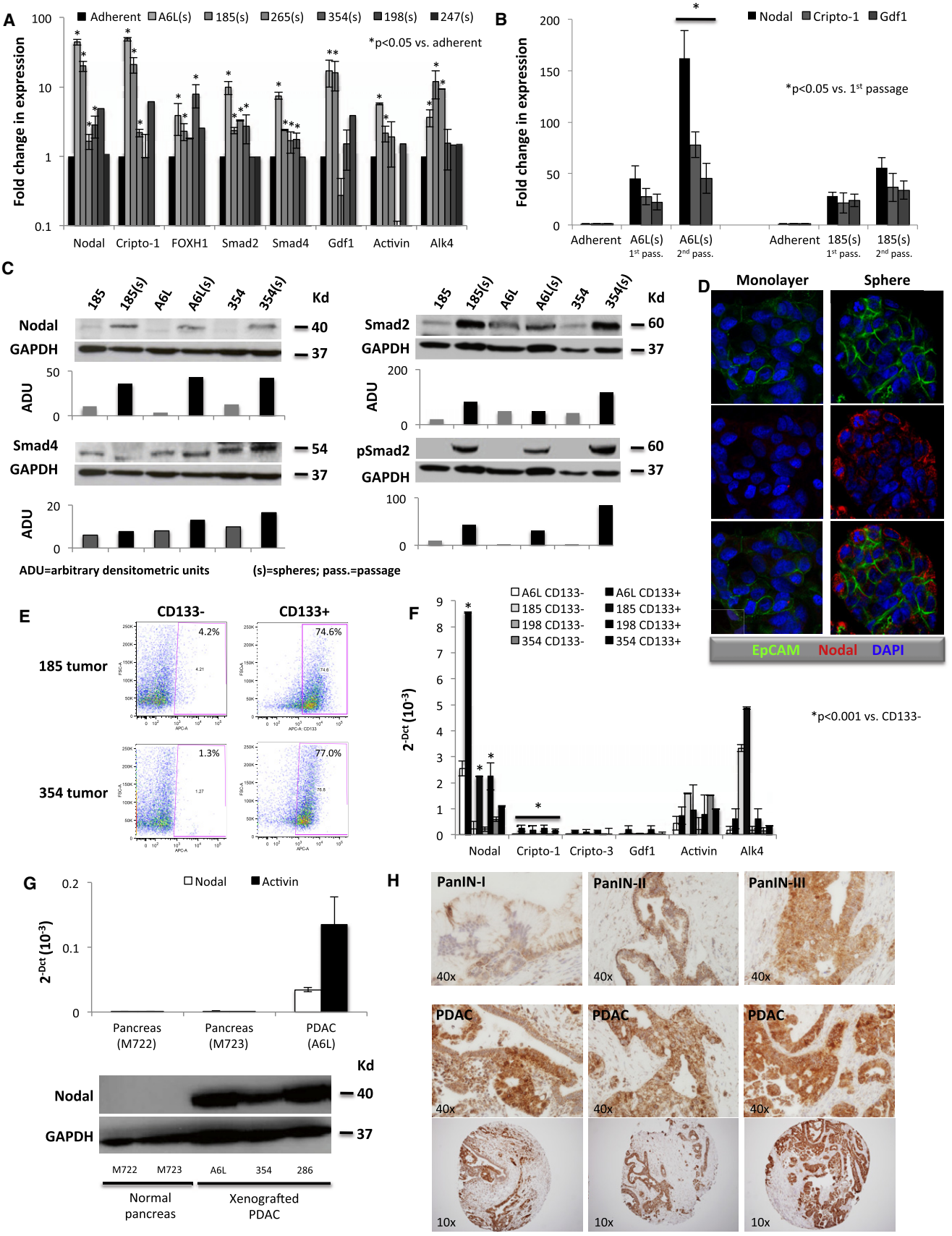
Most importantly, spheres showed enhanced expression of all essential components of the Nodal pathway including phosphorylation of Smad2. This allows its association with Smad4 followed by subsequent translocation to the nucleus to regulate target gene expression, suggesting that the Nodal signaling pathway is operational (Figure 2C). Nodal was hardly detectable in adherent cells by immunohistochemistry, while sphere-derived cells displayed strong cytoplasmic and membranous Nodal protein expression (Figure 2D). Data in established pancreatic cancer cell lines also showed increased expression of Nodal and some of its pathway components in sphere-derived cells, although differences were less pronounced as compared with primary cells, with no difference for the TGF-β signaling pathway (Figures S1D and S1E). We next validated our sphere-based in vitro data using magnetic activated cell sorting (MACS) of CD133$^+$ cells from freshly digested human pancreatic tumor xenografts (185, 198, and 354). Flow cytometry showed good depletion for CD133$^+$ cells in the CD133$^-$ population and revealed enrichment to ~75% in the CD133$^+$ population (Figure 2E). Real-time PCR analysis showed increased expression of Nodal-signaling-associated genes. Specifically, *Nodal*, *Cripto-1*, *Cripto-3*, *Activin*, and *Alk4* were overexpressed in CD133$^+$ cells as compared with CD133$^-$ cells (Figure 2F). Importantly, Nodal expression at the mRNA and protein level was not detectable in normal pancreatic tissue (Figure 2G), but Nodal was highly expressed in pancreatic cancer tissue with strong upregulation during development and progression of primary pancreatic ductal adenocarcinoma as shown in representative tissue microarray samples (Figures 2G and 2H). Because Nodal expression has recently been shown to be reactivated in breast cancer tissue (Topczewska et al., 2006), we also investigated the modulation of Nodal and its pathway components in putative MCF7 breast cancer stem cells (Engelmann et al., 2008). Consistent with the data obtained for pancreatic cancer cells, Nodal, its cofactor Cripto-1, and several pluripotency-associated markers were overexpressed in MCF7-derived spheres as compared with adherent cultures (Figures S1F and S1G). These results indicate that enhanced Nodal expression in the CSC fraction is not restricted to pancreatic cancer.

Nodal/Activin Signaling Is Functionally Active in Pancreatic CSCs

Nodal and Activin are secreted proteins that exert their function by binding to and joining the cell surface receptors Alk4 and Alk7 to form a tertiary ligand-receptor complex that leads to the phosphorylation of Smad2 or Smad3 as intracellular effectors and the subsequent regulation of cell functions. To determine whether rNodal and rActivin, respectively, are capable of activating this signaling cascade, we starved human primary sphere-derived CSCs as illustrated in Figure 3A. Cells were then treated with recombinant protein in the presence or absence of the Alk4/7 inhibitor SB431542 or LY2157299 (specific inhibitor of Alk5). After 30 min of stimulation, phosphorylation of Smad2 and expression of *Nanog*, *Oct3/4*, *Klf4*, *Sox2*, and *Stat3* were determined. Putative cytotoxicity of the utilized inhibitors was excluded by exposure of the cells to the inhibitors for 24 hr followed by DAPI/Annexin V staining (Figure 3B). rNodal/rActivin strongly induced phosphorylation of Smad2, while pretreatment with SB431542 partially abrogated Smad2 phosphorylation after stimulation with rNodal (Figures 3C and 3D). In contrast, TGF-β1 did not induce phosphorylation of Smad2 in sphere-derived cells; nor did the Alk5 inhibitor LY2157299 result in a reduced Smad2 phosphorylation. These results suggest that only Nodal and Activin are capable of activating the Alk4/7 signaling cascade by Smad2 phosphorylation in pancreatic CSCs.

Nodal and Activin Drive Self-Renewal of Pancreatic CSCs

To characterize the biological effects of Nodal and Activin on human pancreatic CSCs, we first examined whether Nodal is capable of enhancing colony formation and self-renewal capacity of pancreatic CSCs. In the sphere formation assay, single cells in suspension were treated with rNodal, rActivin, rTGF-β1, rLefty (endogenous direct inhibitor of Nodal), the Alk4/7 inhibitor SB431542, the specific Alk5 inhibitor LY2157299, and TGF-β receptor II neutralizing antibodies. After 7 days, treatment with rNodal increased the number of spheres as compared with control cells (Figure 4A). In contrast,


A
Adherent
A6L(s)
185(s)
265(s)
354(s)
198(s)
247(s)
*p<0.05 vs. adherent
Fold change in expression
Nodal
Cripto-1
FOXH1
Smad2
Smad4
Gdf1
Activin
Alk4
B
Nodal
Cripto-1
Gdf1
*p<0.05 vs. 1st passage
Fold change in expression
Adherent
A6L(s) 1st pass.
A6L(s) 2nd pass.
Adherent
185(s) 1st pass.
185(s) 2nd pass.
C
185
185(s)
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A6L(s)
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Kd
Nodal
GAPDH
ADU
Smad4
Smad2
pSmad2
ADU=arbitrary densitometric units
(s)=spheres; pass.=passage
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Monolayer
Sphere
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Nodal
DAPI
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185 tumor
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A6L CD133+
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354 CD133+
2-Dct (10-3)
*p<0.001 vs. CD133-
Nodal
Cripto-1
Cripto-3
Gdf1
Activin
Alk4
G
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Activin
2-Dct (10-3)
Pancreas (M722)
Pancreas (M723)
PDAC (A6L)
Kd
Nodal
GAPDH
M722
M723
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286
Normal pancreas
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PanIN-II
PanIN-III
PDAC
40x
10x

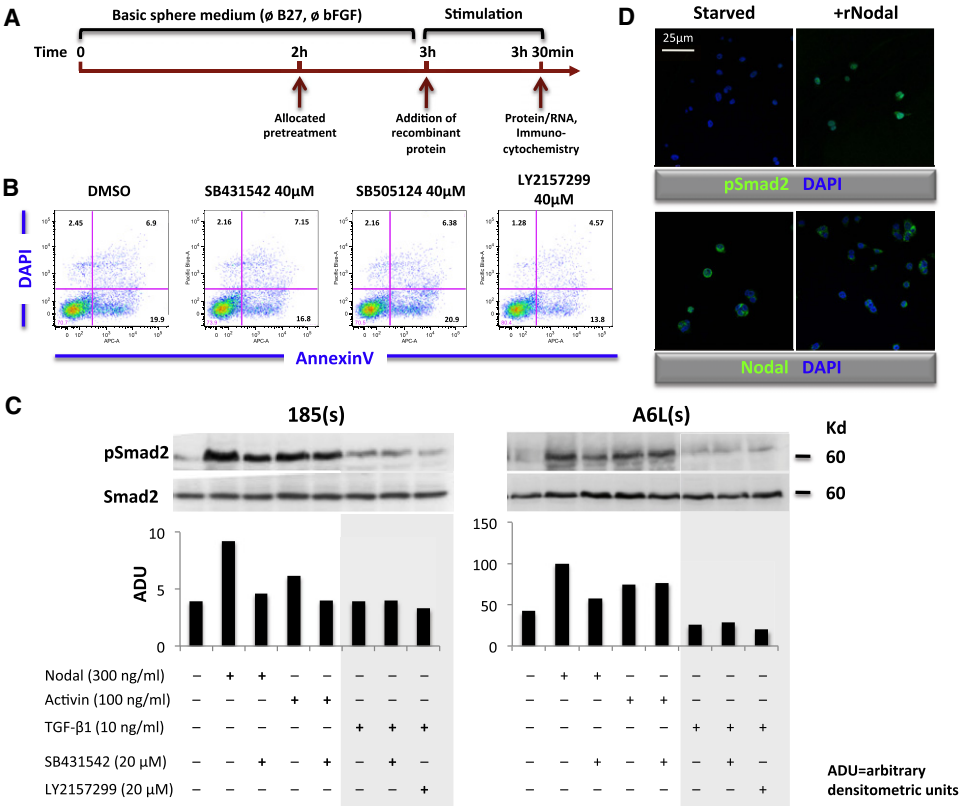

Figure 3. Nodal Signaling Is Functionally Active in Pancreatic CSCs

(A) Schematic illustration of Smad2 phosphorylation assay: cells were kept in basic sphere medium for 3 hr and stimulated for another 30 min with rNodal or rActivin alone or in combination with SB431542. After stimulation molecular and histological analyses were performed.

(B) Cell viability was determined by flow cytometry using DAPI/Annexin V.

(C) Western blotting for pSmad2 and Smad2 after stimulation with rNodal or rActivin alone or in combination with SB431542.

(D) Immunocytochemistry for pSmad2 and Nodal in A6L cells after stimulation with rNodal.

stimulation with rActivin did not result in a profound increase in sphere formation for all tested cells, but in long-term experiments, it did enhance the number of cells with active *Nanog* promoters in sphere cultures to the same extent as Nodal (Figure 4B). The diverse stimulatory effects of rNodal are most likely related to varying levels of endogenous Nodal expression in sphere-forming cells, because pretreatment with the Nodal-specific inhibitor rLefty or the less specific Alk4/7 inhibitor SB431542 dose-dependently (10, 20, and 40 µM) blocked sphere formation, whereas this was not the case in untreated cells (Figure 4A). Consistently, rNodal treatment resulted in the formation of more and larger colonies as compared with control, as determined by a soft agar assay (Figure S2). These data further corroborate the crucial importance of this pathway in the self-renewal capacity of pancreatic CSCs. In contrast, neither the Alk5 inhibitor LY2157299 nor TGF-β receptor II neutralizing antibodies resulted in significant changes in sphere-forming capacity (Figure 4A), suggesting that TGF-β signaling is not relevant for the self-renewal capacity of CSCs.

Next, we investigated the role of the TGF-β family members in the invasive capacity of pancreatic CSCs. A Matrigel-coated, modified Boyden chamber was used to quantitatively evaluate cell invasion. As shown in Figure 4C, the percentage of migrated cells increased significantly after stimulation with rNodal, rActivin, and TGF-β1. Inhibition of Alk4/7 by SB431542 as well as inhibition of Alk5 by SB505124 or the more specific

Figure 2. Components of the Nodal/Activin Signaling Pathway Are Overexpressed in Pancreatic CSCs

qPCR analysis of Nodal-signaling-associated genes in adherent cells versus spheres (s) in first (A) and second passage (B). Data are normalized to GAPDH expression and presented as fold change in gene expression relative to adherent cells. (C) Western blot analysis for Nodal, Smad4, pSmad2, and Smad2 proteins in adherent cells versus spheres. Parallel GAPDH immunoblotting was performed and signal quantification was performed by densitometry. (D) Confocal images for EpCAM (green), Nodal (red), and nuclei (blue) of adherent cells and spheres. (E) CD133 MACS of fresh tumor-derived cancer cells. Purity was validated by flow cytometry using CD133/2. (F) qPCR analysis for Nodal-signaling-associated genes in $CD133^{+}$ cells versus $CD133^{-}$ cells. Data are normalized to GAPDH. (G) qPCR analysis for Nodal and Activin in normal pancreatic tissue from healthy donors (M722 and M723) and pancreatic ductal adenocarcinoma (PDAC). (H) Immunohistochemistry for Nodal (brown) in tissue sections from patients with PanIN-I to PanIN-III lesions and three different patients with PDAC. See also Figure S1.

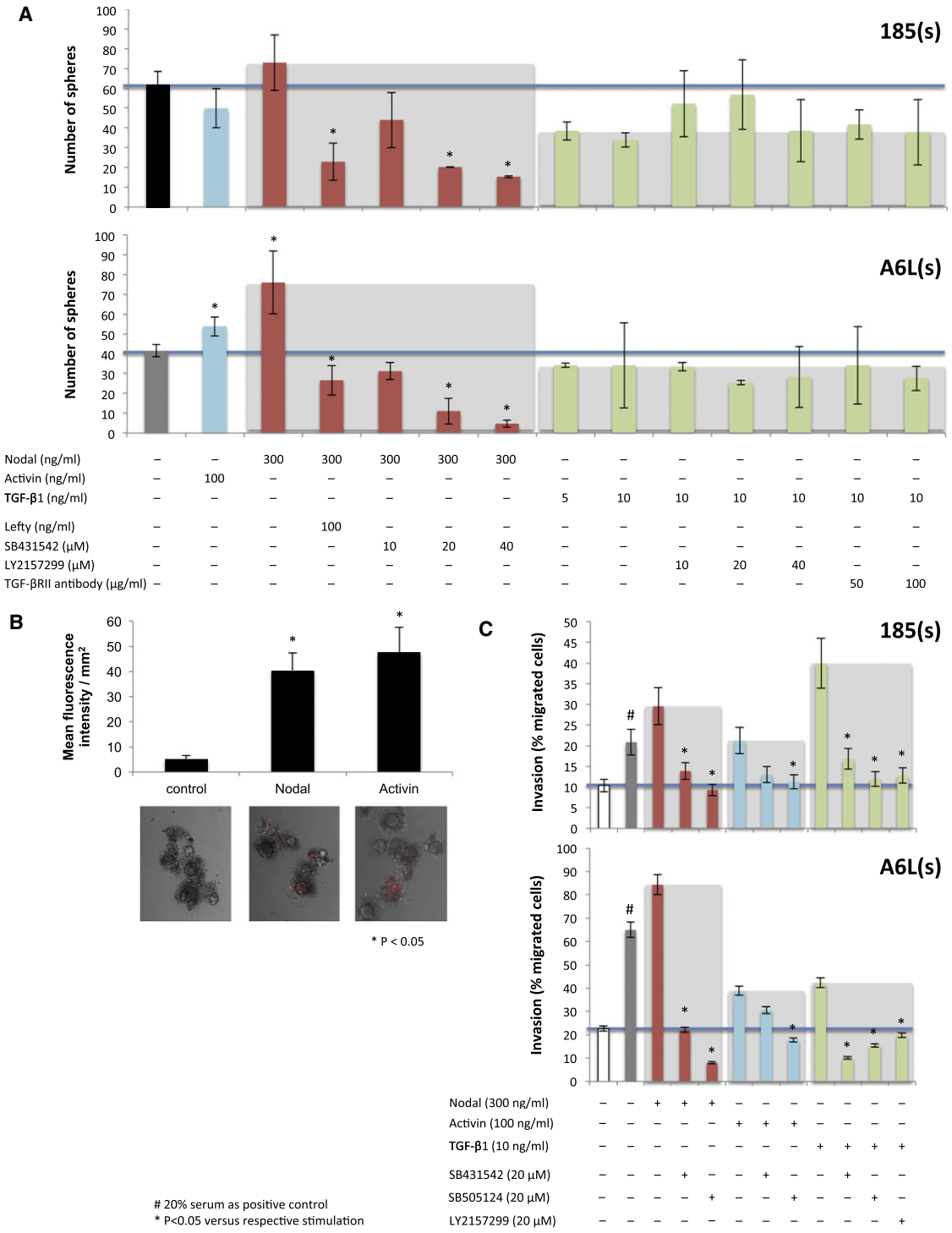

Figure 4. Pharmacological Inhibition of the Nodal/Activin or TGF-β Pathway

(A) Sphere formation capacity of 185 (top panel) and A6L (bottom panel) cells after treatment with the depicted combinations and concentrations of agonists and antagonists of the Nodal/Activin and TGF-β pathways.

(B) A *Nanog* reporter construct expressing RFP was used to detect Nanog promoter^{+} cells (red). Cells were treated with vehicle, rNodal, or rActivin.

(C) Invasion of sphere-derived 185 (top panel) and A6L (bottom panel) cells after treatment as depicted.

See also Figure S2.

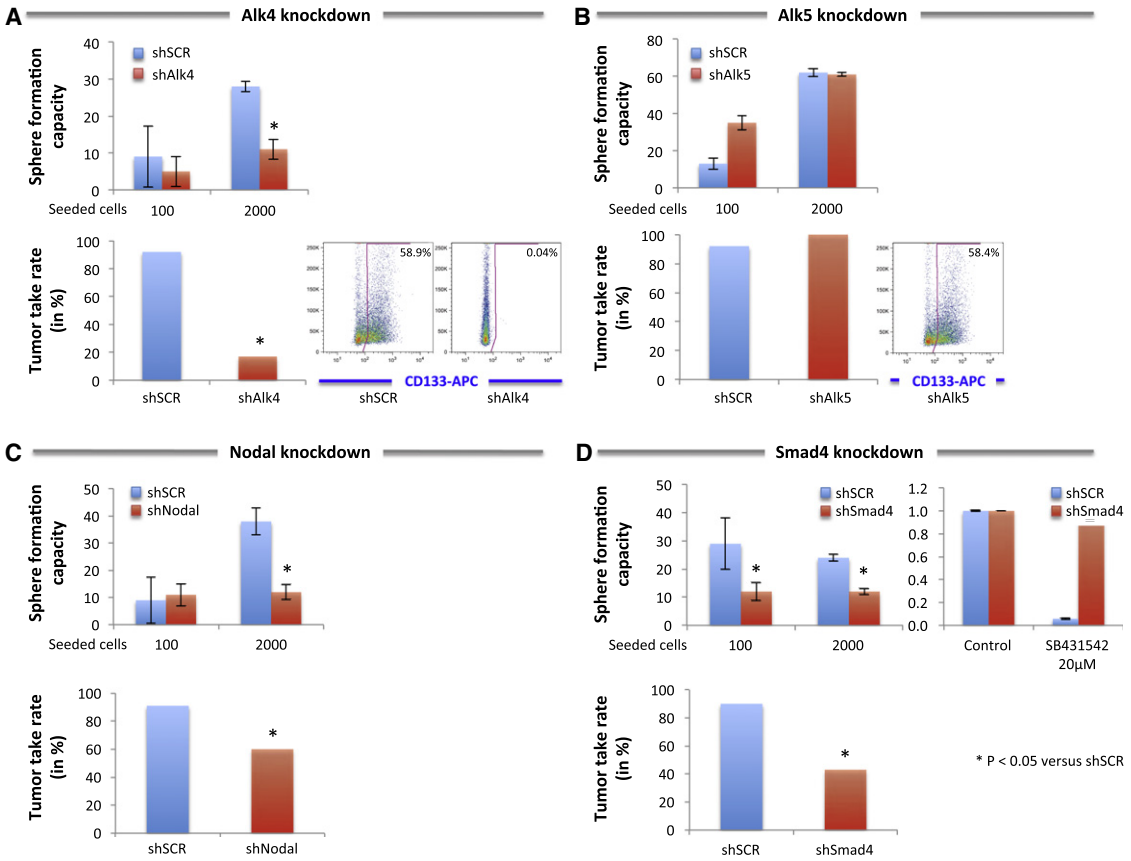

Figure 5. Genetic Targeting of the Nodal/Activin or TGF-β Pathway

(A) Lentivirally transduced cell cultures were sorted by FACS to highest purity based on GFP expression. Comparison of sphere formation capacity (upper panel), in vivo tumorigenicity (lower panel, left), and CD133 content of harvested tumors (lower panel, right) after lentiviral delivery of scrambled or *Alk4* shRNA is shown.

(B) As in (A), but with *Alk5* knockdown.

(C) Lentivirally transduced cell cultures were sorted by FACS to highest purity based on GFP expression. Comparison of sphere formation capacity (upper panel) and in vivo tumorigenicity (lower panel) after lentiviral delivery of scrambled or *Nodal* shRNA is shown.

(D) Lentivirally transduced cell cultures were selected using puromycin resistance. Comparison of sphere formation capacity (upper panel, left), response to SB431542 (upper panel, right), and in vivo tumorigenicity (lower panel) after lentiviral delivery of scrambled or *Smad4* shRNA is shown.

See also Figure S3.

compound LY2157299 completely blocked the enhanced invasiveness of pretreated CSCs. These data indicate that pancreatic CSCs are capable of responding to TGF-β1 by enhanced invasiveness, most likely via Smad2-independent mechanisms (Zhang, 2009).

Finally, the above findings were validated using specific genetic targeting of *Alk4*, *Alk5*, *Nodal*, and *Smad4* using lentiviral delivery of specific shRNA (Table S2 available online). Cells were either selected by FACS for GFP (Alk4, Alk5, Nodal) (Figures S3A–S3C) or by using puromycin resistance (Smad4) (Figure S3D). Knockdown of *Alk4*, which was validated by reduced surface expression of Alk4 as assessed by flow cytometry (Figure S3A), resulted in a significant reduction of sphere formation capacity and, most importantly, drastically reduced in vivo tumorigenicity (Figure 5A). In those few and diminutive tumors that actually formed, $CD133^+$ cells were undetectable. In contrast, knockdown of *Alk5*, also validated by reduced surface expression of Alk5 (Figure S3B), neither affected sphere formation capacity nor resulted in reduced in vivo tumorigenicity (Figure 5B). Consistently, the content of $CD133^+$ cells was not changed as compared with that of scrambled control. Importantly, population doubling of adherent cells was not significantly altered by either knockdown, indicating that these differences were not related to changes in proliferation rate (Figures S3A and S3B). Knockdown of *Nodal* also resulted in significantly lower sphere formation capacity (Figure 5C, Figure S3C). The strong knockdown of *Nodal* translated into significantly reduced in vivo tumorigenicity. Finally, knockdown of *Smad4*, a crucial component of the canonical Alk4/7 signaling cascade, led to a significant reduction in sphere formation capacity (Figure 5D, Figure S3D). Intriguingly, the *Smad4* knockdown translated into reduced in vivo tumorigenicity to a level that was comparable to inhibition of sphere formation and can be rationalized by downstream inhibition of the Nodal/Activin pathway. Indeed, while cells with scrambled shRNA strongly responded to SB431542, cells with knockdown for *Smad4* virtually lost

responsiveness to SB431542 with respect to sphere formation capacity (Figure 5D).

The observation that knockdown of *Nodal* resulted in a less pronounced reduction of in vivo tumorigenicity despite virtually complete knockdown of *Nodal* and strong inhibition of in vitro sphere formation suggests alternative sources for Nodal and/or Activin that may partially overcome the knockdown of *Nodal* in pancreatic CSCs in vivo. Indeed, we found robust expression of Nodal and Activin in human pancreatic stellate cells (PSCs) as an important stromal component of the pancreas (Jesnowski et al., 2005; Ohnishi et al., 2003) (Figure S4A). Sphere formation and invasion of pancreatic CSCs were significantly enhanced by PSC-conditioned medium, an effect that was abrogated by pretreatment with the Alk4/7 inhibitor SB431542 (Figures S4B and S4C). Together with the findings that knockdown of *Nodal* did translate into reduced sphere formation in vitro, but only moderately reduced tumorigenicity in vivo, while knockdown of *Alk4* resulted in strong reduction of both endpoints, we conclude that in vivo CSCs are most likely stimulated in both an autocrine and a paracrine fashion by the stromal compartment.

Nodal Inhibition Chemosensitizes Pancreatic CSCs to Chemotherapy

Pancreatic CSCs are inherently resistant to chemotherapy, resulting in relative enrichment for CSCs in adherent culture as evidenced by flow cytometry (Figure 6A) and an increase in Nodal expression (Figure 6B). Effects were more pronounced in CSC-enriched sphere cultures (Figure 6C). Intriguingly, using CD133 expression as readout for CSC content, we observed a virtually complete elimination of CSCs by inhibiting the Nodal/Activin pathway (Figure 6D), while population doubling was not affected (data not shown). This effect was most consistent in the presence of gemcitabine. These data were validated in freshly isolated patient-derived pancreatic cancer cells (Figure 6E).

For the subsequent investigation of in vivo tumorigenicity of pretreated cells as the most important endpoint, identical numbers of L3.6pl pancreatic cancer cells were exposed to gemcitabine alone, SB431542 alone, or both agents. All surviving cells were orthotopically implanted into the pancreas of immunocompromised mice. No further in vivo treatment was administered. Tumorigenicity was determined by noninvasive PET scan imaging on day 32 and macroscopic and microscopic evaluation on day 35. Importantly, only combination therapy was capable of eliminating in vivo tumorigenicity (Figures S5A–S5C). Mechanistically, we could show that despite a marked decrease in CD133 content following 4 days of treatment with SB431542 alone, the $CD133^+$ population replenished within 48 hr after withdrawal of treatment. When cells were treated with SB431542 and gemcitabine, however, the $CD133^+$ population was irreversibly eliminated (Figure S5D). To further elucidate the mechanism of this finding, we performed cell cycle analyses using BrdU. Treatment with SB431542 alone did not affect the percentage of $CD133^+$ cells in S phase, nor did it increase the percentage of apoptotic $CD133^+$ cells, while the addition of gemcitabine resulted in a 3-fold increase in apoptotic $CD133^+$ cells and virtually complete elimination of cells in S phase (Figure S5E). These findings indicate that SB431542 is capable of reversing the chemoresistance of the tumorigenic CSC population, most likely by (reversibly) driving them into a more differentiated state as evidenced by temporary loss of CD133.

Nodal/Activin Inhibition in Established Pancreatic Cancers

Based on these promising findings, we then investigated whether inhibition of Nodal/Activin by SB431542 translates into increased progression-free survival in pre-established pancreatic cancers. Because only the combination pretreatment with gemcitabine resulted in loss of tumorigenicity in vivo, we focused on this treatment regimen. Xenografts were established by orthotopic implantation of L3.6pl cells into athymic mice and treatment was started 1 week after injection. The detailed experimental setup is depicted in Figure 7A. Harvesting of some tumors after the last round of SB431542 administration revealed efficient in vivo targeting of the Nodal/Activin pathway with subsequent downregulation of Nodal (Figures S6A–S6D). Tumor growth was assessed on day 42 by palpation and confirmed by magnetic resonance imaging (MRI) (Figure 7B). No tumors were detectable in mice receiving combination therapy, so the study was continued until day 100 to monitor progression/putative relapse of disease. Over time, control animals bore large, life-limiting tumors and succumbed within 40 days after tumor implantation (median survival time: 32 days). Gemcitabine alone significantly prolonged survival due to inhibition of tumor growth, but all animals showed progressive disease with median survival still severely limited with 54 days. For the combination of SB431542 and gemcitabine, long-term survival was significantly better compared with gemcitabine alone, with 100% survival at day 100 (Figure 7C).

Next, we investigated the effects of SB431542 in primary human pancreatic cancer tissue xenografts as the ultimate preclinical setting (see study design in Figure 7D). In contrast to the above findings for implantation of cancer cells, the addition of SB431542 to gemcitabine treatment did not result in a deceleration of growth of primary tumor tissue (Figure 7E). During long-term follow-up, it was only when gemcitabine was already withdrawn that tumor growth eventually started to slow down, as compared with tumors treated with gemcitabine alone, while the latter actually reaccelerated in growth. This difference in response to gemcitabine withdrawal resulted in a modest, but significantly reduced, tumor burden at the 100 day follow-up point.

Based on this rather disappointing outcome and stimulated by data from Olive et al. (2009), we hypothesized that this modest treatment effect could be attributed to poor drug delivery in stroma-rich primary pancreatic cancer tissue. Indeed, mass spectrometry analysis revealed that SB431542 was hardly detectable in tumor-bearing mice after 2 weeks of treatment (Figure S6E). Intriguingly, the addition of the Smoothened inhibitor CUR199691 (CUR) for targeting the hedgehog pathway in stromal cells drastically improved drug delivery by 10-fold. Therefore, we next tested a triple combination therapy (gemcitabine, SB431542, and CUR), which translated into immediate inhibition of tumor progression and eventually translated into long-term stable disease at 100-day follow-up (Figures 7E and 7F and Figure S7A). Histological evaluation of the tumors explanted by the end of the study confirmed a marked depletion of tumor stroma in the triple therapy group, as well as a higher grade of differentiation, although the later changes were of a more subtle nature (Figure 7F). Cells isolated from harvested

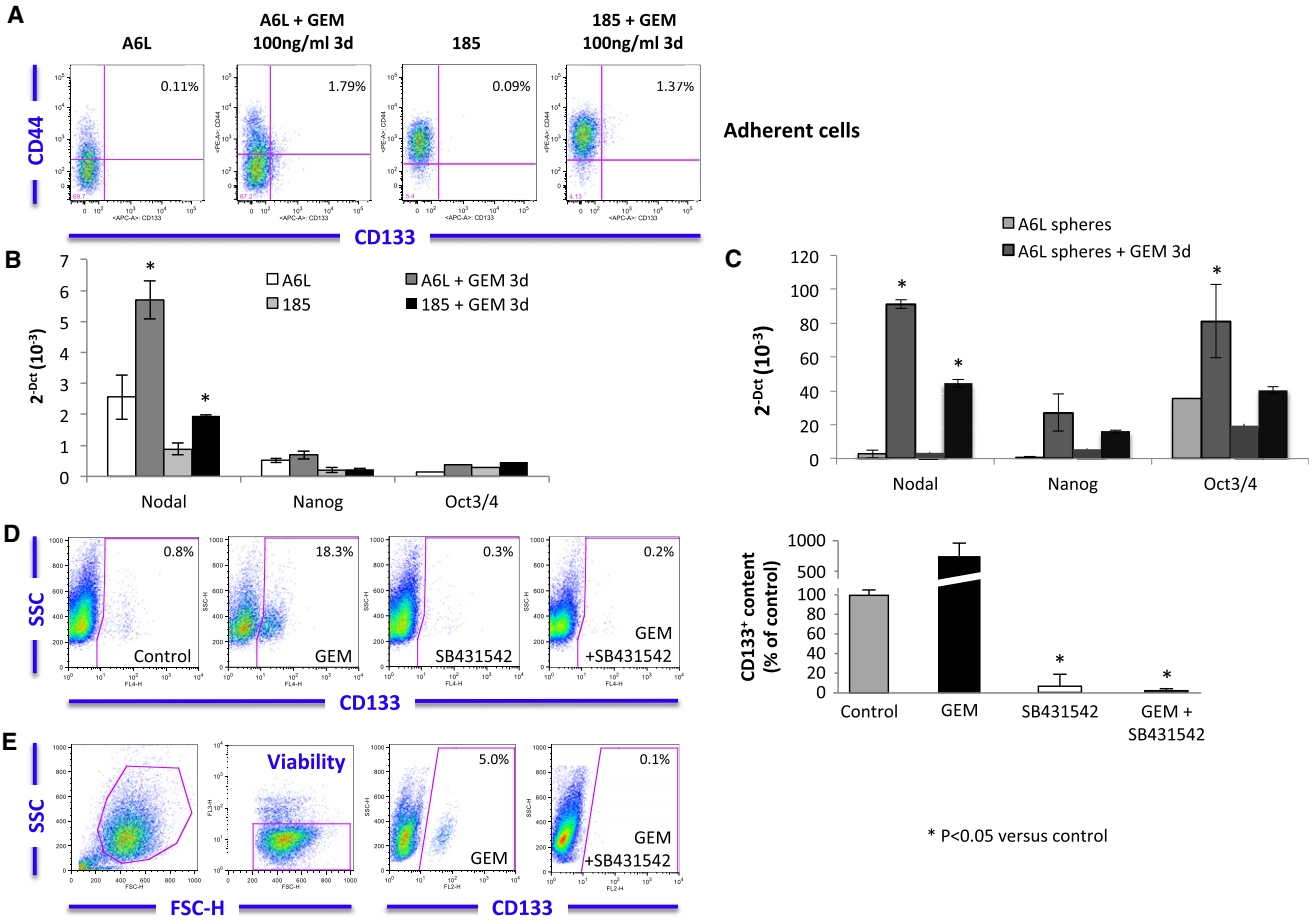

Figure 6. Nodal Inhibition Targets CD133$^+$ CSCs

(A) Flow cytometry for CD44 and CD133 in A6L and 185 adherent cells untreated or treated with gemcitabine (GEM). qPCR analysis for *Nodal*, *Nanog*, and *Oct3/4* genes in A6L and 185 adherent cells (B) or spheres (C) untreated or treated with GEM is shown. Data are normalized for GAPDH expression. (D) Flow cytometry for CD133 expression in L3.6pl cells untreated or treated as indicated (left panel). Quantification of CD133 expression in respective groups, with n = 3 (right panel), is shown; data are mean ± SEM, n ≥ 3. (E) Flow cytometry for CD133 in freshly isolated primary human pancreatic cancer cells treated with GEM in the presence or absence of SB431542. See also Figure S4.

tumors treated only with gemcitabine demonstrated strong sphere-forming capacity as opposed to cells isolated from tumors treated with gemcitabine plus SB431542, which already showed a significant reduction in sphere-forming capacity (Figure 7G, left panel). Most intriguingly, however, cells derived from tumors treated with triple therapy had virtually lost their sphere-forming capacity. Consistent patterns were observed for phenotyping of the cells using flow cytometry. CD133$^+$ or CD133$^+$/CD44$^+$ cells were significantly reduced in the group receiving triple combination therapy (Figure 7G, right panel). Consistently, administration of triple therapy to another tumor (JH051) resulted in similar treatment response. Taken together, these data demonstrate that triple therapy is capable of eliminating tumor-promoting pancreatic CSCs in vivo, leading to long-term progression-free survival.

DISCUSSION

Patients with pancreatic ductal adenocarcinoma are still suffering from a devastating prognosis, which can be at least partially rationalized by the observation that the standard chemotherapeutic agent gemcitabine is not capable of eliminating CSCs. Indeed, gemcitabine rather leads to a relative increase in the number of CSCs, indicating a preferential targeting of more differentiated and rapidly proliferating cancer cells. The restricted elimination of the more differentiated cancer cells, even if associated with significant tumor size reduction, will not lead to the eradication of the tumorigenic potential of the tumor, as that is restricted to the CSC population. Here we demonstrate that the Nodal/Activin pathway is essential for the self-renewal capacity and stemness properties of pancreatic CSCs. Nodal/Activin is strongly expressed in pancreatic CSCs, but is also expressed by PSCs, which are abundantly present in the stroma surrounding pancreatic cancer cells and may serve as a CSC niche.

In a large set of primary cells and (fresh) primary patient tissues, we then showed that the CSC compartment is severely affected by inhibition of this pathway by making use of three different approaches: first, by using a small molecule inhibitor (SB431542) targeting the Nodal/Activin receptor Alk4; second,

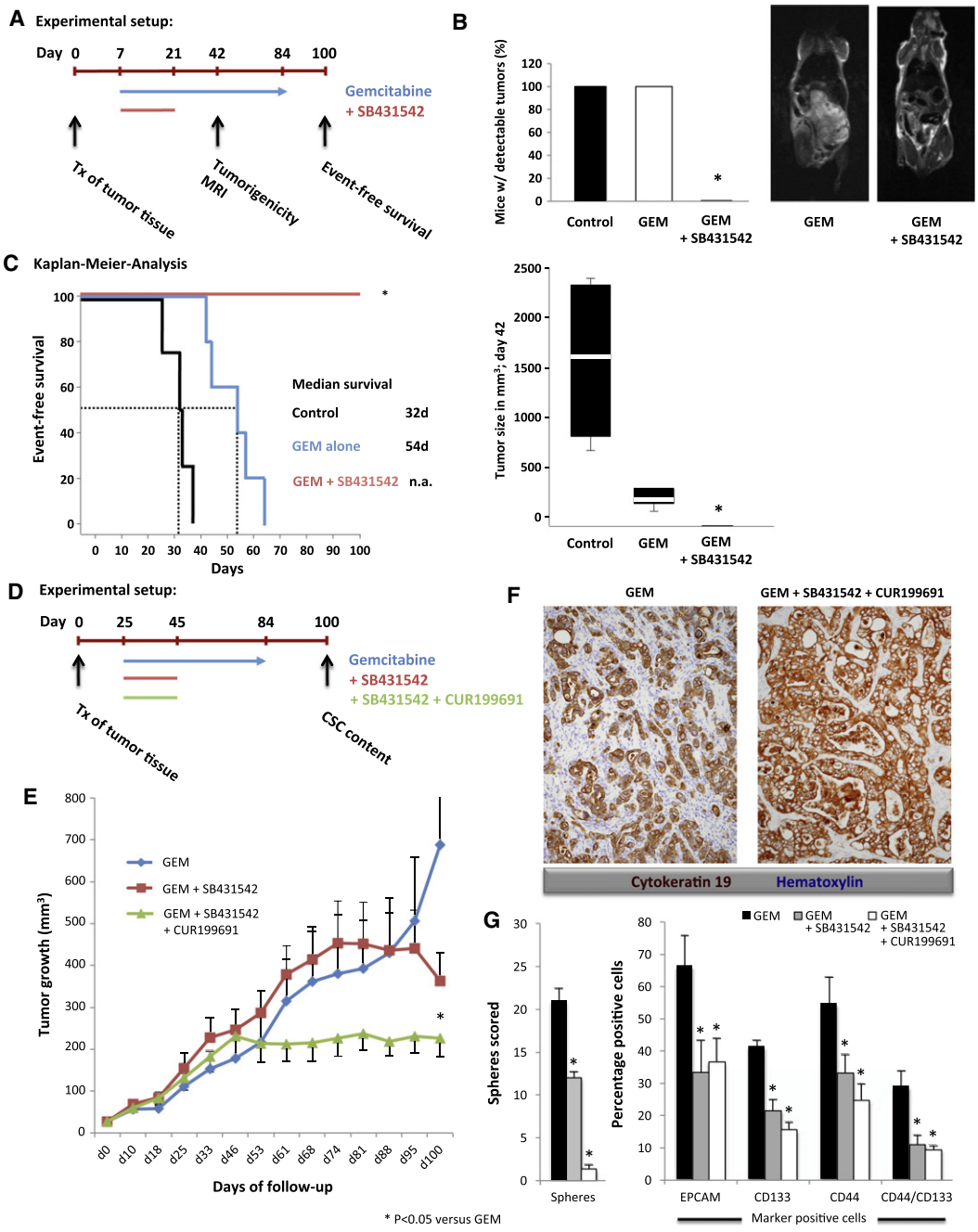

Figure 7. In Vivo Effects of Nodal/Activin Inhibition on Established Pancreatic Cancers

(A) Experimental setup for in vivo experiments using L3.6pl cells.

(B) Tumor take-rate (upper left panel), tumor size (lower panel), and representative MRI pictures (right panel) of treated pancreatic cancers.

(C) Kaplan-Meier analysis depicting cumulative survival of respective treatment groups; data are mean ± SEM, n ≥ 3.

(D) Experimental setup for in vivo experiments using primary human pancreatic cancer tissue.

(E) Tumor growth is depicted for the respective treatment groups. Data are mean ± SEM, n ≥ 3.

(F) Histological evaluation on day 100 using cytokeratin 19 staining for gemcitabine alone (GEM) and triple-treated tumors as indicated.

(G) On day 100, tumors from the different groups were digested and analyzed for their respective sphere formation capacity (left panel) and cell surface marker expression (right panel); data are mean ± SEM, n ≥ 3.

See also Figures S5–S7.

by using recombinant Lefty as the specific endogenous Nodal inhibitor; and third, by genetic knockdown of *Nodal*, *Alk4*, and *Smad4* using shRNA technology. Our findings are in line with earlier observations that have identified other developmental pathways such as mTOR, hedgehog, Notch, and BMP for targeting CSCs (Bar et al., 2007; Li et al., 2007; Mueller et al., 2009;

Piccirillo and Vescovi, 2006), although their targeting may be of limited clinical use for at least some of them due to normal stem-cell-related side effects. Intriguingly, an important feature of the herein described Nodal/Activin pathway is its complete lack of activity in normal pancreas and other adult tissue (Topczewska et al., 2006), spurring the hope for little to no side effects because normal stem cells will most likely be spared.

Nodal and Activin are involved in developmental biology by perpetuating the undifferentiated state of ESCs (Vallier et al., 2005; Xiao et al., 2006). While the expression of Activin and the Nodal coreceptor Cripto-1 have previously been demonstrated in pancreatic cancers (Friess et al., 1994; Kleeff et al., 1998), we here provide evidence that Nodal, the second ligand of the Alk4/7 receptor, is expressed in this malignancy, but not in normal pancreas. Most importantly, Nodal is capable of strongly propagating the tumorigenic CSC subpopulation as demonstrated by its pharmacological inhibition using the extracellular Nodal antagonist Lefty and shRNA technology, whereas Activin was less drastically enriched in pancreatic CSCs and showed limited effects on their self-renewal capacity in some tumors. These data are in line with previous reports showing that Nodal is crucial for tumorigenicity in melanoma and breast cancer cells, with an embryonic microenvironment reducing tumorigenic activity and inducing the expression of epithelial markers by the secretion of Lefty (Postovit et al., 2008; Topczewska et al., 2006).

On the other hand, Activin reportedly contributes to an invasive phenotype in esophageal carcinoma, another epithelial malignancy (Yoshinaga et al., 2004, 2008). In a previous report on the dynamic regulation of the invasive phenotype of breast cancer cell lines, the interconversion from noninvasive epithelial-like $CD44^+CD24^+$ cells to invasive mesenchymal $CD44^+CD24^-$ progeny was also found to be Nodal/Activin dependent (Meyer et al., 2009). Consistently, we now provide evidence that Activin also promotes invasion of pancreatic CSCs as does Nodal. These data have important implications because they indicate that therapeutic strategies should not focus on either Nodal or Activin, but rather focus on Alk4/7 as their common receptor. Indeed, a comprehensive set of experiments proves that targeting this pathway by blocking the Alk4/7 receptor using the small molecule inhibitor SB431542 and shRNA technology has a strong impact on both the $CD133^+$ fraction that is enriched for CSCs and sphere formation capacity.

Next, we identified human PSCs as an important component of the stroma that also strongly expresses Nodal/Activin. Conditioned medium from PSCs promoted self-renewal and invasiveness of pancreatic CSCs. PSCs, which reside in exocrine areas of the pancreas, are myofibroblast-like cells known to be activated upon insult. These cells are analogous to hepatic stellate cells, with which they share 99% identity at the transcriptome level (Omary et al., 2007). PSCs are important mediators in the pancreatic response to injury because they migrate to the damaged location and promote cell proliferation, migration, and assembly (Shimizu, 2008). Therefore, because our data suggest that PSCs may represent an in vivo niche for CSCs, targeting these interactions could be of pivotal importance for the development of more effective therapies for pancreatic cancer. While targeting Alk4/7 as the common receptor for Nodal/Activin should abrogate autocrine and paracrine signaling, directly eliminating this paracrine source for Nodal/Activin may provide additional therapeutic benefits. Intriguingly, this can be achieved by targeting the hedgehog pathway as a crucial signaling component for PSCs (Bailey et al., 2008; Shinozaki et al., 2008), and may account, at least in part, for the striking therapeutic effects generated by the addition of a smoothened inhibitor to our armamentarium for treating primary pancreatic cancer tissue in our studies.

However, translating our findings into the in vivo setting was not only challenged by alternative sources for Nodal/Activin, but also by the fact that the Nodal/Activin small molecule inhibitor SB431542 as a single therapy was not sufficient to irreversibly eliminate the cells' ability to form tumors in vivo. This lack of in vivo translation of the apparently encouraging in vitro effects could be explained by the enhanced plasticity of pancreatic cancer cells. Indeed, after withdrawal of SB431542 and continued culture of the cells, a drastic rebound of the $CD133^+$ population was also observed in vitro, which retrospectively rationalizes the still-preserved in vivo tumorigenicity of the cells. However, the rebound of $CD133^+$ CSCs upon withdrawal was prevented by addition of gemcitabine to the treatment regimen. Further mechanistic studies revealed that SB431542 alone (reversibly) drives CSCs into a more differentiated state, as evidenced by loss of CD133, but cells still retain the ability to revert to the CSC phenotype. Intriguingly, although gemcitabine alone led to a relative enrichment of CSCs, the combination of SB431542 and gemcitabine resulted in their irreversible and complete elimination. Indeed, in vitro combination therapy resulted in complete abrogation of the in vivo tumorigenic potential of the remaining cells.

This chemosensitizing effect of SB431542 should be of great therapeutic value for patients with pancreatic cancer and was therefore further evaluated in vivo. However, testing this treatment regimen in mouse models of pancreatic cancer came with another caveat. Our first in vivo experiments in established pancreatic cancer, which were based on the orthotopic implantation of isolated pancreatic cancer cells, confirmed the in vitro data by illustrating robust therapeutic efficacy and 100% survival at 100 day follow-up for SB431542 plus gemcitabine. Surprisingly, however, when we then moved to a preclinical model using xenografted primary human pancreatic cancer tissue, tumor development remained virtually unaffected by this combination. It is important to note that xenografted pancreatic cancer tissues contain large amounts of stroma whereas implantation of cancer cells regularly lacks this important feature. Tumor-associated stroma does not only provide an additional source for Nodal/Activin as described above, but is also capable of modulating tumor vascularization, which could interfere with drug delivery to cancer (stem) cells. Indeed, impaired drug delivery has already been demonstrated for pancreatic cancer in a recent landmark study using a genetically engineered mouse model (Olive et al., 2009).

Therefore, breaching the "stroma fortress" of pancreatic cancer represents an important challenge for drug delivery in general (Neesse et al., 2010) and CSC-targeted therapies in particular because these cells have been proposed to preferably reside in hypoxic niches (Borovski et al., 2011; Heddleston et al., 2009). Intriguingly, when we coadministered the hedgehog pathway inhibitor CUR199691 (Mueller et al., 2009) to deplete

the stromal compartment together with SB431542, we observed a 10-fold increase in drug delivery into the tumor tissue. The addition of gemcitabine then translated into rapid disease stabilization, and none of the mice required sacrificing during the 100 day study period. Failure to completely eradicate the remaining small tumors can be rationalized by the lack of response of nonproliferating tumor cells to gemcitabine. Most importantly, however, these small lesions no longer contain CSCs; cells isolated from these remnant tumors did not form spheres anymore. In contrast, all mice treated with gemcitabine alone had to be sacrificed within 100 days due to excessive tumor growth. Cells isolated from these tumors bear strong sphere forming capacity. Therefore, our data demonstrate the successful combination of stroma- and CSC-targeting strategies for effectively treating pancreatic cancer in most relevant preclinical models.

Canonical downstream signaling of Alk4/7 is mediated by Smad2/3 as well as the Co-Smad Smad4, which is shared by all TGF-β family members. Importantly, about 50% of patients with pancreatic cancer bear inactivating mutations or deletions of the *Smad4* gene, which could result in dysfunction of the pathway (Schneider and Schmid, 2003). While noncanonical TGF-β family signaling pathways have been described and may account for the enhanced TGF-β1-induced invasiveness of pancreatic CSCs (Zhang, 2009), we found that *Smad4* knockdown in previously Smad4-competent cells resulted in reduced in vivo tumorigenicity, most likely via inhibition of Nodal/Activin signaling, because these cells no longer responded to the Alk4/7 inhibitor SB431542. Therefore, because Smad4 seems indispensable for the Nodal/Activin signaling cascade, tumors carrying functionally relevant *Smad4* mutations or deletions may not respond to a Nodal/Activin-targeting therapy. Importantly, however, not all *Smad4* mutations actually result in dysfunctional Smad4; we have identified several tumors bearing *Smad4* mutations that still demonstrate a functional Smad2/3 cascade, including a subsequent translocation of pSmad2 into the nucleus, and that respond to this triple therapy. Future studies will have to address the question of which patients will most likely respond to this treatment modality and how best to identify them.

EXPERIMENTAL PROCEDURES

Primary Human Pancreatic Cancer Cells

Human pancreatic tumors were obtained with written informed consent from all patients. For in vitro studies, tissue fragments were minced, enzymatically digested with collagenase (Stem Cell Technologies, Vancouver, BC) for 60 min at 37°C (Mueller et al., 2009), and, after centrifugation for 5 min at 1200 rpm, resuspended as pellets and cultured in RPMI, 10% FBS, and 50 units/ml penicillin/streptomycin.

Pancreatic cancer spheres were generated and expanded in DMEM:F12 (Invitrogen, Karlsruhe, Germany) supplemented with B-27 (GIBCO, Karlsruhe, Germany) and bFGF (PeproTech EC, London, UK). Ten thousand cells per milliliter were seeded in ultra-low attachment plates (Corning B.V., Schiphol-Rijk, Netherlands) as described previously (Gallmeier et al., 2011). After 7 days of incubation, spheres were typically >75 μm large with ~97% $CD133^{high}$. For serial passaging, 7-day-old spheres were harvested using 40 μm cell strainers, dissociated to single cells with trypsin, and then regrown for 7 days. Cultures were kept no longer than 4 weeks after recovery from frozen stocks (passage 3–4).

Human Pancreatic Cancer Cell Lines

The human pancreatic cancer cell lines L3.6pl, Panc1, and MiaPaCa2 were maintained as previously described (Hermann et al., 2007).

In Vivo Treatment of Established Pancreatic Cancers

Single-cell suspensions were either orthotopically implanted into the pancreas of female nude mice (Harlan Europe), or 2 mm^3 pieces of primary, in vivo expanded pancreatic cancer tissue were subcutaneously implanted and mice were randomized to the respective treatment groups. Size and weight of the pancreatic tumors were monitored. Gemcitabine was administered twice a week (125 mg/kg i.p.). SB431542 was used at 25 mg/kg, and CUR199691, at 100 mg/kg, both by oral gavages twice daily for 3 weeks.

Cytometry

To identify pancreatic CSCs, the following antibodies were used: anti-CD133/1-APC or PE (Miltenyi, Bergisch-Gladbach, Germany); anti-CXCR4-APC, anti-SSEA-4-FITC, SSEA-1-APC, EpCAM-FITC, and CD44-PE (all from Beckton Dickinson, Heidelberg, Germany); and anti-Alk4, anti-Alk5, and anti-Cripto-1-PE (all from Cell Signaling Technology, Inc.); or appropriate isotype-matched control antibodies. CD133/2-APC (Miltenyi) was used for purity testing after MACS. Propidium iodide, 7-AAD, or DAPI was used for exclusion of dead cells (eBiosciences, San Diego, CA). Samples were analyzed by flow cytometry using a FACS Canto II (BD) and data were analyzed with FlowJo 9.2 software (Ashland, OR).

Immunofluorescence

Primary pancreatic cancer cells and spheres were seeded in 96-well dishes (Corning, NY) and incubated at 37°C for 3 hr. Cells were washed with cold PBS and then fixed with prechilled 4% PFA for 20 min at room temperature. After blocking with 1% bovine serum albumin in PBS-Triton 0.1%, cells were incubated with primary antibodies: Nodal (ab556676; Abcam, Inc.), pSmad2 (3108; Cell Signaling), and EpCAM (BD) overnight at 4°C in the dark. Then cells were washed three times with PBS-Triton 0.1% and incubated with Alexa-Fluor-conjugated secondary antibodies against mouse or rabbit (Invitrogen) at room temperature for 1 hr in the dark. Cells were mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA) and analyzed using an SP5 confocal microscope (Leica, Heidelberg, Germany).

Western Blot Analysis

PVDF membranes containing electrophoretically separated proteins from human primary pancreatic cancer cells and spheres were probed with mouse antibodies against Oct4a (2890), Smad2 (3103; both Cell Signaling Technology), Nanog (ab21624), Nodal (ab556676), GAPDH (ab8245-100; all Abcam), Smad4 (sc-7966; Santa Cruz Biotech), or rabbit antibody against pSmad2 (3108; Cell Signaling), treated with peroxidase-conjugated goat anti-mouse or anti-rabbit Ig secondary antibody (Sigma), and then visualized by enhanced chemiluminescence (Amersham).

Smad2 Phosphorylation Assay

Human primary sphere-derived single cells were grown for 3 hr in DMEM:F12 (GIBCO) without bFGF and B27. Following starvation, cells were incubated for 30 min at 37°C with recombinant human rNodal, Activin, or TGFb1 (R&D) either alone or in the presence of SB431542 (Sigma) or LY2157299 (Axon MedChem, Groningen, Netherlands. Anti-Smad2 and anti-phospho-Smad2 (Ser465/467, Cell Signaling) antibodies were used following the manufacturer's instructions.

RNA Preparation and Real-Time PCR

Total RNAs from human primary pancreatic cancer cells and spheres were extracted with TRIzol kit (Life Technologies Inc.) according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis with SuperScript II reverse transcriptase (Life Technologies Inc.) and random hexamers. Quantitative real-time PCR was performed using SYBR Green PCR master mix (QIAGEN), according to the manufacturer's instructions. The list of utilized primers is depicted in Table S1.

Invasion and Migration Assays

Invasion assays were performed using modified Boyden chambers filled with Matrigel (BioCoat, BD Biosciences, Heidelberg, Germany). Cells were pretreated with SB431542, SB505124, LY2157299, TGF-β receptor II neutralizing antibodies, or recombinant human Lefty for 1 hr. Five hundred microliters of cell suspensions containing 5×10^4 pretreated or untreated cells were added to the Matrigel-coated inserts, and seven hundred and fifty microliters of

serum-free medium with or without recombinant human Nodal, recombinant human Activin, or recombinant human TGF-β1 were added to the lower chamber. The assay chambers were incubated for 22 hr at 37°C. Invaded cells were fixed in 4% PFA and stained with DAPI. The ratio of cells in the lower chamber versus total seeded cells was calculated.

Lentiviral shRNA Delivery

As lentiviral shuttle backbone we used a pLVX shRNA2 plasmid (Clontech). shRNA constructs were generated by hybridization in solution of HPLC-purified paired oligonucleotides with the recessed restriction sites (BamHI and EcoRI) added to the sequence for cloning purposes. As control we used pLVX-shRNA expression vectors encoding a scrambled shRNA sequence with no target (in silico prediction). The shRNA sequences were selected from the RNAi Consortium website (www.broadinstitute.org/rnai/public). The inserts of shRNA were annealed from sense and antisense oligonucleotides with the sequences as provided in Table S2. Lentivirus production and titration were carried out as previously described (Torres et al., 2011) and regularly contained 1×10^7 T.U./ml with a 1:100 T.U./physical particles ratio as quantitated by qPCR. Cells were then transduced with lentiviral stocks diluted to an M.O.I. of 50 in the presence of polybrene (8 μg/ml, Sigma). A6L and 185 cells were seeded at a density of 5×10^4 cells per 24 multiwell plate and allowed to adhere overnight. The next day, cells were infected with the lentivirus for 6 hr. Stably transduced cells were obtained after cell sorting for GFP included in the viral vector (for *Alk4*, *Alk5*, *Nodal*) or using puromycin resistance (*Smad4*). For the transduction with the Nanog promoter reporter, we used a human Nanog-RFP construct with a zeomycin resistance marker for System Biosciences (SBI; Mountain View, CA).

MRI

Mice were analyzed with a 3-Tesla MRI system (Magnetom Tim Trio, Siemens, Erlangen, Germany) using a dedicated small animal coil and T2-weighted scanning.

Statistical Analyses

Results for continuous variables are presented as means ± standard deviation (SD) unless stated otherwise. Treatment groups were compared with the independent samples t test. Pair-wise multiple comparisons were performed with the one-way ANOVA (two-sided) with Bonferroni adjustment. $p < 0.05$ was considered statistically significant. All analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, Illinois).

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes two tables and seven figures and can be found with this article online at doi:10.1016/j.stem.2011.10.001.

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Multimodal treatment eliminates cancer stem cells and leads to long-term survival in primary human pancreatic cancer tissue xenografts

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Pancreatic cancers contain exclusively tumourigenic cancer stem cells (CSCs), which are highly resistant to chemotherapy, resulting in a relative increase in CSC numbers during treatment with gemcitabine as standard chemotherapeutic agent. In this project we have further consolidated our previous findings that $CD133^+$ pancreatic cancer stem cells are highly resistant to chemotherapy, and show that CSCs have an increased hedgehog and mammalian Target of Rapamycin (mTOR) pathway activity. The separate use of inhibitors of these (stem cell) pathways resulted only in a slight reduction of the $CD133^+$ cell fraction. However, the combined use of a hedgehog pathway inhibitor (SIBI-C1) and an mTOR inhibitor (Rapamycin) together with the cytotoxic agent Gemcitabine (= SIBI+R+Gem) resulted in a virtually complete elimination of CSCs in primary human pancreatic cancers.

Therefore we embarked on further studies evaluating this new therapeutic regimen in a clinically most relevant model using mice with pre-established xenografted human pancreatic tumours. The resulting triple treatment indeed led to a successful depletion of the cancer stem cell pool, and additionally also markedly changed the cellular composition of the tumours, depleting the supportive stroma. Overall, this resulted in long-term survival of the mice while control animals treated with chemotherapy alone died within a few weeks. Since we have previously also performed a large-scale pre-clinical safety study, we now firmly establish this (= SIBI+R+Gem) triple therapy as a novel therapeutic option for the treatment of advanced pancreatic adenocarcinoma.

I contributed to the design of this study, performed the experiments together with the other authors, analyzed and interpreted the results. I also participated in the writing of the manuscript, with input from the rest of the authors and under the supervision of the thesis director Prof. Christopher Heeschen.

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Multimodal Treatment Eliminates Cancer Stem Cells and Leads to Long-Term Survival in Primary Human Pancreatic Cancer Tissue Xenografts

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Abstract

Purpose: In spite of intense research efforts, pancreatic ductal adenocarcinoma remains one of the most deadly malignancies in the world. We and others have previously identified a subpopulation of pancreatic cancer stem cells within the tumor as a critical therapeutic target and additionally shown that the tumor stroma represents not only a restrictive barrier for successful drug delivery, but also serves as a paracrine niche for cancer stem cells. Therefore, we embarked on a large-scale investigation on the effects of combining chemotherapy, hedgehog pathway inhibition, and mTOR inhibition in a preclinical mouse model of pancreatic cancer.

Experimental Design: Prospective and randomized testing in a set of almost 200 subcutaneous and orthotopic implanted whole-tissue primary human tumor xenografts.

Results: The combined targeting of highly chemoresistant cancer stem cells as well as their more differentiated progenies, together with abrogation of the tumor microenvironment by targeting the stroma and enhancing tissue penetration of the chemotherapeutic agent translated into significantly prolonged survival in preclinical models of human pancreatic cancer. Most pronounced therapeutic effects were observed in gemcitabine-resistant patient-derived tumors. Intriguingly, the proposed triple therapy approach could be further enhanced by using a PEGylated formulation of gemcitabine, which significantly increased its bioavailability and tissue penetration, resulting in a further improved overall outcome.

Conclusions: This multimodal therapeutic strategy should be further explored in the clinical setting as its success may eventually improve the poor prognosis of patients with pancreatic ductal adenocarcinoma.

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Competing Interests: Two of the authors are currently (Patrizia Tunici) or were until recently (Annette Bakker) employed by the commercial funder of this research 'Siena Biotech S.p.A'. Patrizia Tunici and Annette Bakker developed and validated SIBI-C1, but were not involved in the design and data analysis of the present study. The authors also confirm that this does not alter their adherence to all the PLOS ONE policies on sharing data and materials. No other relevant declarations are made relating to employment, consultancy, patents, products in development or marketed products, etc.

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Introduction

Pancreatic ductal adenocarcinoma (hereafter referred to as "pancreatic cancer" or PDAC) is the fourth most frequent cause of cancer-related death world-wide [1,2,3] and is characterized by a high rate of metastasis and pronounced resistance to chemotherapy and radiation. Despite extensive research efforts over the past decades, little substantial progress has been made towards improving clinical endpoints [4]. Although the introduction of the anti-metabolite gemcitabine in 2007 has improved clinical response by reducing pain and weight loss [5], disease prognosis has remained extremely poor with a 5 year survival rate of ~3–4% and a median survival period of 4–6 months [1,6]. Indeed, several studies have consistently shown that gemcitabine treatment mostly targets differentiated cancer cells resulting in a relative enrichment of cancer stem cells [7,8,9]. For patients with metastatic disease, but good performance status, the recent combination therapy FOLFIRINOX (oxaliplatin, irinotecan, fluorouracil, and leucovorin) showed a significant survival advantage but with increased

toxic side-effects [10]. Alternatively, the regimen of nab-paclitaxel plus gemcitabine showed substantial anti-tumor activity with more tolerable adverse effects in a phase I/II trial, warranting phase III evaluation [11]. However, in all these trials the majority of the patients ultimately succumbed from disease progression. Thus, the development of new anti-cancer therapeutics and/or new treatment modalities remains a high healthcare priority.

With increasing evidence supporting the existence of cancer stem cells, a new horizon is emerging in the development of therapeutic strategies against pancreatic cancer. Cancer stem cells represent a subpopulation of cells distinguishable from the bulk of the tumor based on their exclusive ability to drive tumorigenesis and metastasis. These cells also play a crucial and driving role in disease relapse [12,13,14,15,16]; therefore, the elucidation of the mechanisms underlying pancreatic tumorigenesis and especially pancreatic cancer stem cells is of crucial relevance for the development of more efficient clinically-available therapies. Indeed, we have recently developed novel approaches that both target cancer stem cells and overcome their mechanisms of chemoresistance [9,17,18]. For example, we have shown that the self-renewal capacity of pancreatic cancer stem cells is dependent on both *Hedgehog* and *mTOR* signaling, and simultaneous targeting of these two pathways, in combination with Gemcitabine, represents a novel treatment strategy for epithelial cancers such as pancreatic cancer [9]. Building on these studies, we here investigate the applicability, safety, and potential for further optimization of this combination therapy approach in a large set of primary patient-derived tumors.

Results

Triple Therapy Markedly Reduces Tumor Size and Increases Survival

We have shown previously that sphere cultures of pancreatic cancer cells enrich for cancer stem cells [8,9,17], and that combined targeting of the Sonic Hedgehog (SHH) and mTOR pathways may offer a new therapeutic option. Here we verify in four distinct primary pancreatic cancer cell lines derived from patient tumors that cancer stem cell-enriched sphere cultures indeed show marked overexpression of SHH and the Hedgehog target genes GLI-1 and GLI-2 (**Fig. 1A**), as well as increased mTOR pathway activity (**Fig. 1B**). The subsequent *in vivo* evaluation of the combination therapy was performed in clinically most relevant models of patient-derived pancreatic cancer whole-tissue xenografts (see **Fig. 1C** for study design). Pieces of briefly *in vivo* expanded primary human pancreatic tumors containing heterogeneous populations of cancer cells including cancer stem cells [9] as well as stromal cells [7], pancreatic stellate cells, inflammatory cells, and extracellular matrix were implanted subcutaneously and orthotopically into immunocompromised mice. Tumor take rate was confirmed by tumor growth during two successive size measurements, and tumor-bearing mice were randomized for treatment. Subsequently, the tumors were measured once weekly either by caliper (subcutaneous tumors) or with a small-animal ultrasound imaging system (orthotopic tumors). As Gemcitabine (Gem) represents the current standard treatment for pancreatic cancer, we used Gem-treated mice as the reference group.

A set of representative tumors was selected based on their diverse response to Gem treatment [7]. PDAC-265 and 185 were highly resistant to Gem treatment, showing rapid tumor growth so that the first mice had to be removed from the study within 3 weeks of the start of the treatment (**Fig. 2A–E**) due to excessive tumor growth. In contrast, in tumors PDAC-JH051, 247, and Pax22, Gem treatment resulted in initial treatment response and disease stabilization; however, after the removal of chemotherapy, the tumors reproducibly started to re-grow (**Fig. 2C–E**). Of all the tumors investigated, only PDAC-354, which does not carry *Kras* mutations [19], showed significant response to Gem treatment until the end of the observation period (**Fig. 2F**) and closely mimicked the treatment response observed in the actual patient (data not shown).

Importantly, we were able to improve treatment response by combining chemotherapy with the novel hedgehog pathway inhibitor SIBI-C1 (SIBI; Siena Biotech) [20]. SIBI strongly inhibits gene expression of SHH and downstream target genes such as GLI2 in primary pancreatic cancer cells *in vitro* (**Fig. S1A in File S1**). SIBI was administered for only 3 weeks to reduce potentially deleterious effects. Gem was given for a total time period of 60 days in accordance with common clinical practice (**Fig. 1C**). Due to the strong response to chemotherapy alone, co-treatment of mice bearing PDAC-354 xenografts with either SIBI did not show an additional effect at the level of tumor size (**Fig. 2F**) or survival (data not shown). For all of the other tumors, however, double treatment with Gem+SIBI led to a marked reduction in tumor size (dashed line, **Fig. 1 & 2**), significant delay in tumor growth, and thus significantly prolonged survival compared to mice receiving either no treatment or Gem+Vehicle (**Fig. 2G**). Importantly, however, tumors eventually relapsed limiting survival in mice receiving this double therapy. These data are in line with improved delivery of gemcitabine following depletion of protective stromal tissue [21]. As previously shown, inhibition of hedgehog signaling alone does not completely abrogate the cancer stem cell population (**Fig. 3A & B**) [**9**].

Since we have shown in comprehensive *in vitro* studies that cancer stem cells can indeed be eliminated by the addition of an inhibitor of the mTOR pathways [9], we next investigated the effect of a treatment regimen comprising Gem, SIBI, and established mTOR inhibitor rapamycin (Rapa) on our representative panel of primary pancreatic cancer tissue xenografts. Interestingly, we observed a very strong response to this triple therapy, resulting in disease stabilization or even regression in almost all tumors investigated (dotted line, **Fig. 1 & 2**). This translated into a significantly improved cumulative survival as compared to all other treatment groups (**Fig. 2E**).

Combination Therapy Depletes Cancer Stem Cell Content and Alters Tumor Composition

In order to evaluate the *in vivo* effects of combination therapy on cancer stem cell populations, we explanted and digested representative tumors of each group after completion of the 3 weeks of triple therapy, and analyzed by flow cytometry the expression of surface markers previously linked to a cancer stem cell phenotype [8,9,13]. The percentage of $EpCAM^{+}CD133^{+}CD44^{+}$ cells in Gem-treated tumors was regularly 2-3-fold higher as compared to untreated tumors [data not shown and [8]]. In contrast, Gem+SIBI already showed a slight decrease in cancer stem cell numbers as compared to Gem alone (**Fig. 3A & B**). Importantly, only the addition of Rapa to the treatment regimen virtually eliminated cancer stem cells from the tumor. Furthermore, upon termination of the study period (day 200) we investigated secondary sphere formation as a functional assay for cancer stem cell activity in PDAC-Pax22 tumors and observed that sphere formation capacity was slightly diminished for cultures derived from tumors treated with Gem+SIBI as compared to Gem alone. Interestingly, however, it was only after triple treatment that we could observe complete abrogation of sphere formation activity

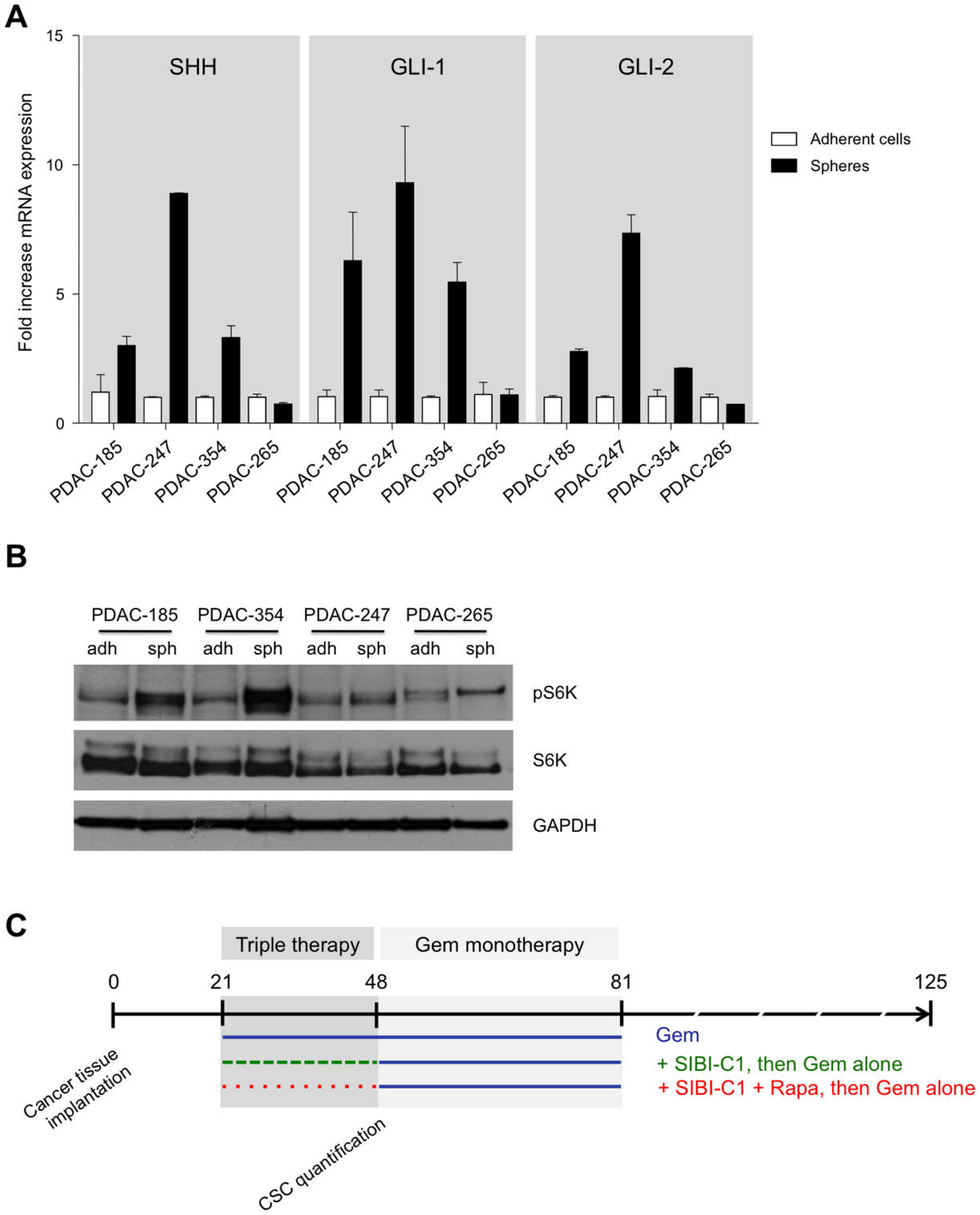

Figure 1. Targeting of sonic hedgehog and mTOR in pancreatic ductal adenocarcinoma. (**A**) Fold increase mRNA expression levels of SHH, GLI-1, and GLI-2 of sphere-derived vs. adherent cells. (**B**) Western blot analysis of mTOR pathway activity via the assessment of S6 kinase expression (upper panel) and phosphorylation (lower panel) in adherent primary cells versus stem cell-enriched sphere-derived cells. (**C**) Illustration of experimental setup. Duration of triple therapy is marked by a dark grey box (day 21 to 48), Gem monotherapy with a light grey box (day 48 to 81).
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(**Fig. 3C**), suggesting that triple combination therapy had effectively depleted the cancer stem cell pool in the tumor.

More detailed histological investigation of the tumors showed that the different treatment regimens also modified the cellular composition of the tumor. While the primary tumor-derived

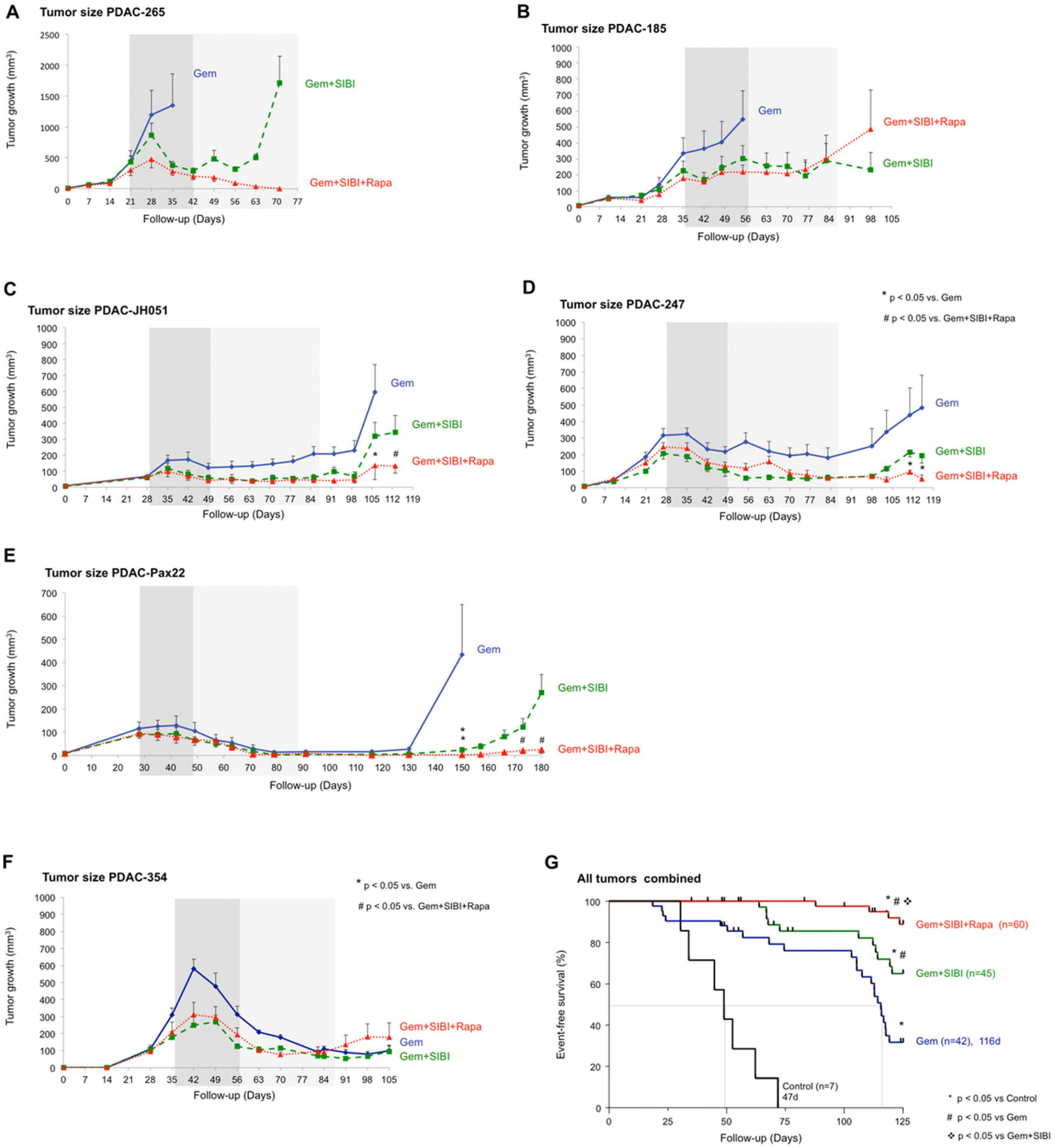

Figure 2. Combination therapy in a representative set of pancreatic ductal adenocarcinoma. (**A–F**) Tumor growth curves for primary whole-tissue xenografts PDAC-265, PDAC-185, JH051, 247, Pax22, and 354 implanted subcutaneously and orthotopically. Continuous line depicts Gem+vehicle, dashed line depicts Gem+SIBI, dotted line depicts Gem+SIBI+Rapa (n≥6 per group). (**G**) Kaplan-Meier Curve depicting cumulative survival time of all mice pooled by treatment group.
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xenografts used for this study displayed a reasonably high amount of stroma (35–70%) in the groups treated with Gem only (**Fig. 4A, left panels**), the addition of a hedgehog pathway inhibitor markedly decreased the stroma content (**Fig. 4A, middle panels**), an observation that is well in line with previous published reports [21]. This effect was slightly more pronounced after the addition of Rapa (**Fig. 4A, right panels**), and was statistically significant as compared to tumors treated with Gem alone (**Fig. 4B**). As expected, we observed the same effects after treatment of Gem-sensitive tumors (**Fig. S1B in File S1**). Interestingly, we observed similar effects in orthotopic tumors (**Fig. 4A**) as in subcutaneous tumors (**Fig. S1B in File S1**).

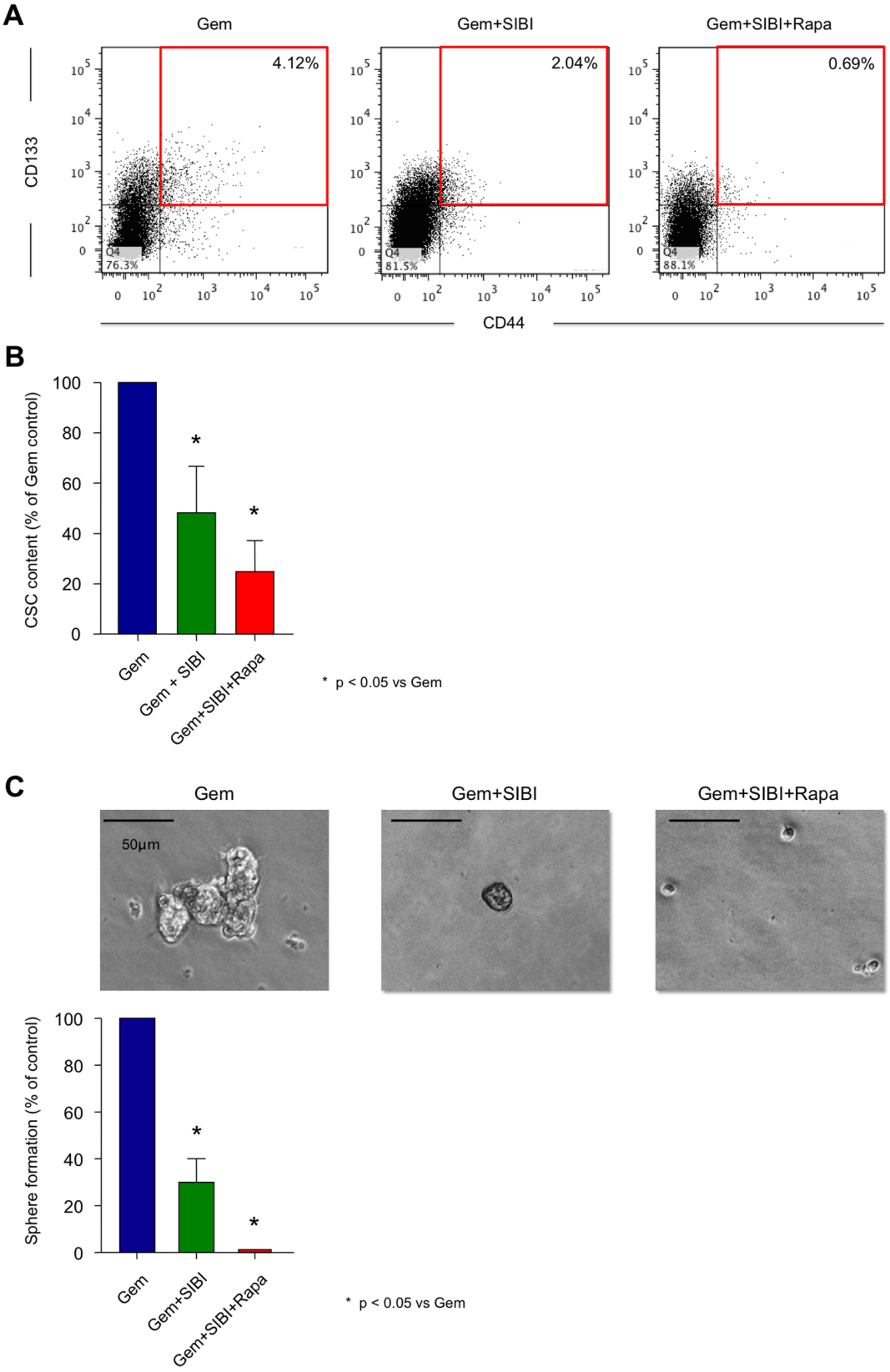

Figure 3. Effect of combination therapy on cancer stem cell content. (**A**) Representative flow cytometry plots and (**B**) quantification of cancer stem cell ($EpCAM^{+}CD133^{+}CD44^{+}$) content of tumors in the respective treatment group (cumulative results of cells obtained from different xenografts). (**C**) Representative images and quantification of secondary sphere formation of treated PDAC-Pax22 tumors explanted at the end of the experiment (d200).
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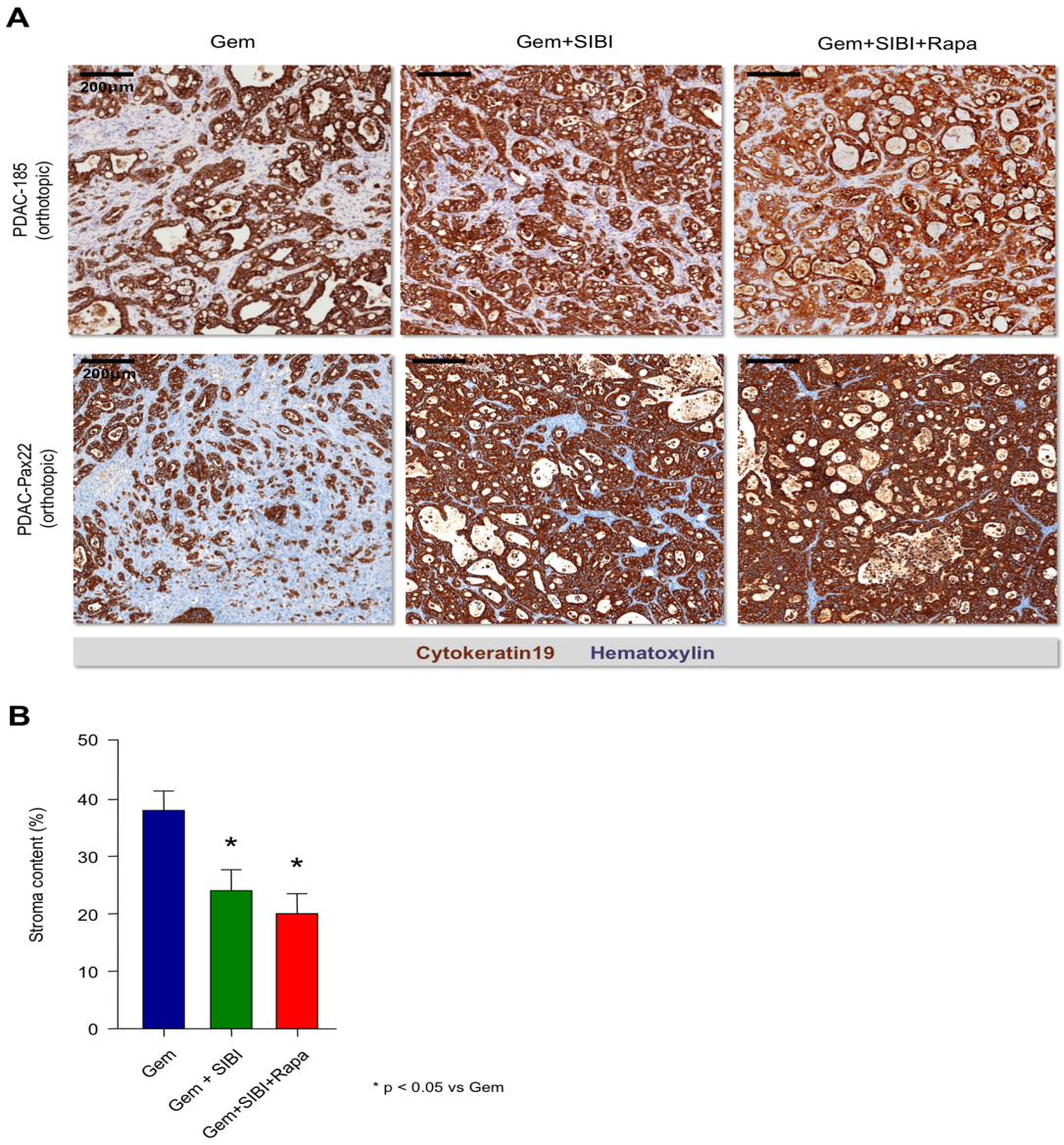

Figure 4. Effect of combination therapy on tumor composition. (**A**) Representative histological pictures showing stroma content in the respective treatment groups in gemcitabine resistant orthotopic tumors (**PDAC-185, upper panel**), (**Pax22, lower panel**). (**B**) Quantification of stroma content throughout the different treated xenografts.
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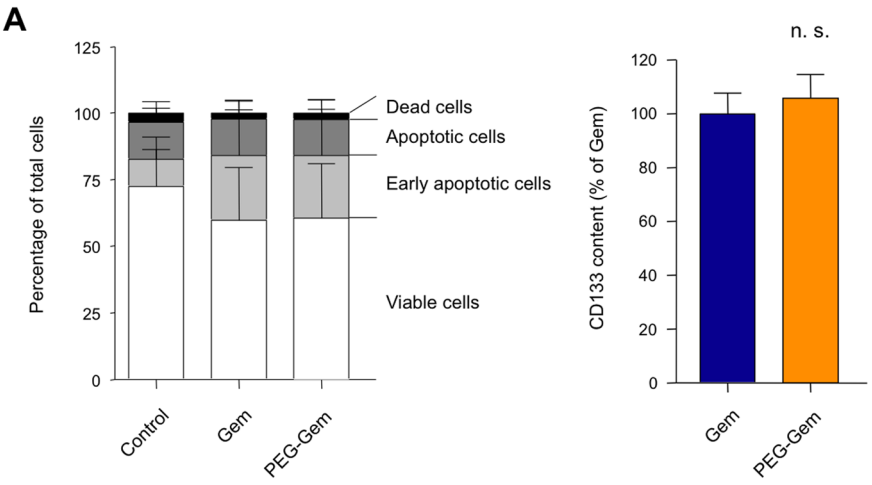
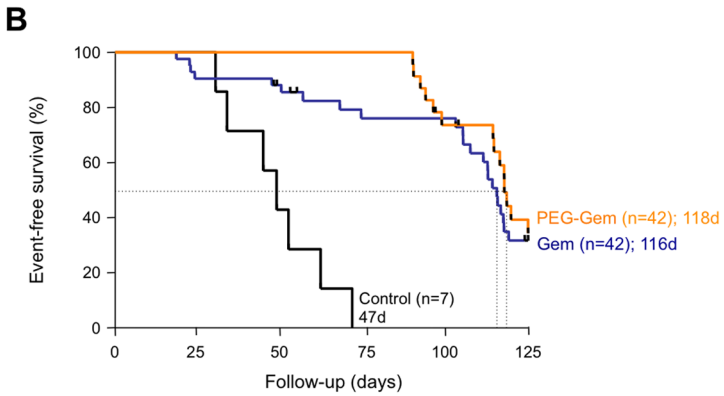
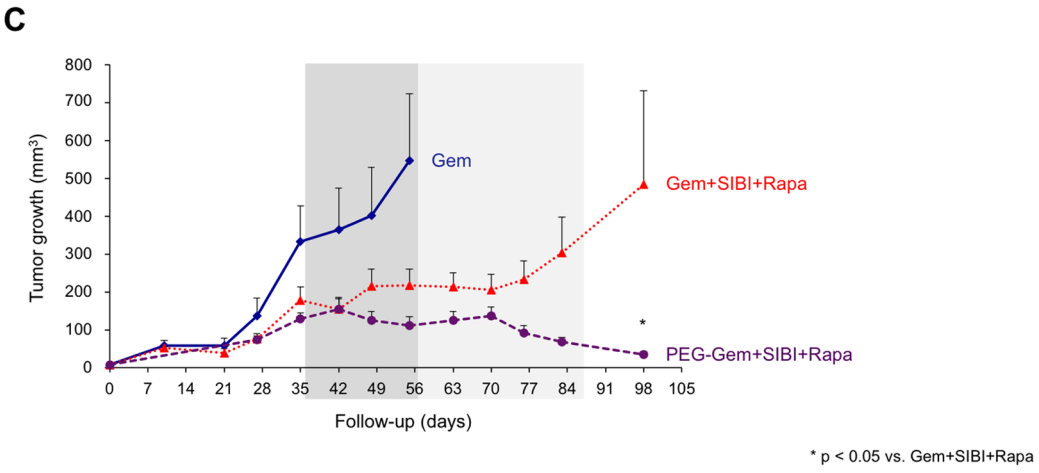
PEGylation of Gemcitabine further Enhances the Effects of Combination Therapy

Since previous reports have shown that modifying the chemical structure of Gem by PEGylation leads to significantly increased circulation time and tissue penetration *in vivo* and may therefore be a novel option for the improved treatment of patients with (pancreatic) cancer [22,23], we decided as a next step to determine the effects of PolyEthyleneGlycol-bound Gem (PEG-Gem) as the extended *in vivo* circulation time and higher tissue penetration of PEG-Gem may generate superior effects as compared to standard Gem. First, we evaluated the *in vitro* effects of PEG-Gem as compared to Gem on freshly isolated primary human pancreatic cancer cells. For this purpose, four matching primary cell cultures generated from *in vivo*-expanded pancreatic cancer tissues were treated for 48 hours with either standard Gem or PEG-Gem and were subsequently analyzed by flow cytometry for the induction of apoptosis or cell death, as well as for their cancer stem cell content. Regarding the percentage of apoptotic and dead cells, no differences could be observed between treatment groups (**Fig. 5A left panel, and data not shown**). In addition, we did not observe differences between standard Gem and PEG-Gem treatment regarding the content of $CD133^+$ cells *in vitro* (**Fig. 5A right panel**).

Next we treated mice bearing orthotopic or subcutaneous primary tumor-derived whole-tissue xenografts with PEG-Gem, analogous to the treatment regimen for standard Gem. We selected tumors that showed insufficient response with Gem. While we did not observe a significant difference for median survival between PEG-Gem and standard Gem for these tumors (**Fig. 5B**), it is important to note that the onset of tumor-related death in mice treated with PEG-Gem was far later than with standard Gem (Time until progression: PEG-Gem 91d vs. Gem 19d). Encour-







* p < 0.05 vs. Gem+SIBI+Rapa

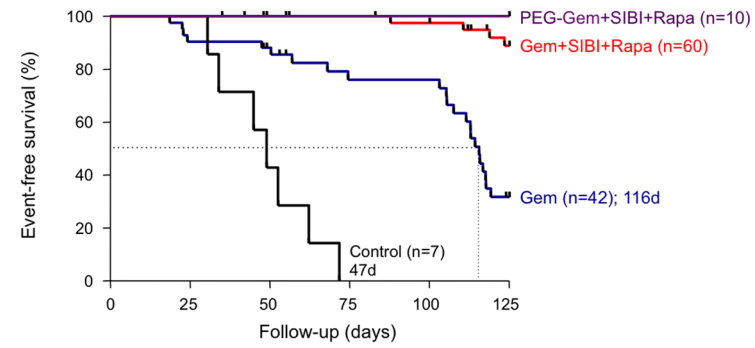


Figure 5. Comparison of the *in vitro* and *in vivo* effects of Pegylated Gemcitabine. (**A**) *In vitro* effects of Gem and PEG-Gem on apoptosis and cell death as well as CD133 expression (cumulative results of cells obtained from different xenografts). (**B**) Kaplan-Meier Curve depicting cumulative survival time of all mice pooled by treatment group. For illustrative purposes, selected survival curves of Fig. 2D are depicted again. (**C**) Tumor growth curves for primary whole-tissue xenografts implanted subcutaneously and orthotopically, respectively. Continuous line depicts Gem+vehicle, dashed line depicts Gem+ SIBI, dotted line depicts Gem+SIBI+Rapa. (**D**) Kaplan-Meier Curve depicting cumulative survival time of all mice pooled by treatment group. For illustrative purposes, selected survival curves of Fig. 2D are depicted again.
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aged by these promising results, we next replaced standard Gem treatment with PEG-Gem in the triple therapy approach (PEG-Gem+SIBI+Rapa) and evaluated the effects on tumor growth and survival in mice bearing PDAC-185 patient-derived xenografts. While in our initial *in vivo* studies Gem+SIBI+Rapa treatment led to significantly reduced tumor growth and short-term sustained disease as compared to standard Gem treatment (**Fig. 2D**), many tumors eventually relapsed. In response to treatment with PEG-Gem+SIBI+Rapa, however, we observed virtually complete regression of the tumors (**Fig. 5C**), which resulted in 100% survival until the end of the observation period (day 125) (**Fig. 5D**).

Combination Therapy Shows no Significant Toxicity

Potential toxicity remains a major concern for combination therapy approaches. To assess cumulative toxicity of the administered treatments and their respective combinations, we recorded body weight for all treated mice on a weekly basis, starting on the day of randomization until day 100. Excluding cachexia as a potential treatment-induced side effect, no significant differences in body weights were observed between the treatment groups (**Fig. 6A**), Furthermore, in order to exclude potentially deleterious effects on the function of normal stem cells (e.g. in the hematopoietic system), we additionally monitored white blood cell numbers in the treated mice at the completion of the 3 weeks of single versus combined therapies. While at this point of the study the expected cumulative toxicity would be the highest, no significant reduction in white blood cell counts was observed in any of the treatment groups as compared to standard Gem treatment ($p = 0.792$) (**Fig. 6B**), suggesting no extensive alterations of hematopoietic stem cells by the triple combination treatment. Interestingly, even the increased circulation time and improved tissue penetration of PEG-Gem did not significantly increase expected adverse side effects as compared to standard Gem treatment (**Fig. 6A & B**).

Discussion

Here we validate the concept of a multimodal therapy for comprehensively targeting the diverse cell compartments in pancreatic cancer using a representative set of almost 200 subcutaneous and orthotopic whole-tissue primary tumor xenografts, making this one of the largest investigations in the cancer stem cell field. Tumors were selected based on their previously described diverse response to gemcitabine treatment [7]. Chemotherapy and radiation primarily target differentiated cancer cells, and while these therapies induce apoptosis and cell death in tumor cells, a population of cancer stem cells is highly resistant [8,9,18,24], survives the standard therapy, and maintains the ability to re-populate a tumor in all its heterogeneity. Double treatment combining Gem and the new Smoothened inhibitor SIBI consistently prolonged survival in mice transplanted with tumors. Importantly, however, only in mice treated with triple therapy cancer stem cells were virtually completely abrogated, and we observed a long-term disease stabilization or regression, and subsequent long-term survival. In this combination regimen, the treatment effect of conventional Gem could be further enhanced by the use of PEGylated Gem via enhancing its bioavailability.

At the histological level, pancreatic cancer is characterized by very dense stroma and poor vascularization. Olive et al. showed in a genetically engineered mouse model of pancreatic cancer that the stroma is strongly dependent on hedgehog signaling, and inhibition of the hedgehog pathway with smoothened inhibitors leads to "preferential" killing of stromal cells and increased vessel density [21], thus making tumor cells more accessible to therapeutic intervention. While these observations were obtained in a mouse model of pancreatic cancer, we have more recently shown that also in patient-derived whole-tissue xenografts co-treatment with a smoothened inhibitor significantly increases drug delivery [17] and markedly reduces tumor-associated stroma formation. Importantly, we now appreciate that the stroma not only hampers drug delivery [21], but also provides a supportive niche for cancer stem cells promoting their self-renewal capacity and invasiveness [25]. Thus, elimination or abrogation of the stroma does significantly improve treatment regimens by distinct mechanism, but is only capable of eliminating cancer stem cells if combined with Gem.

However, despite the rather modest response of cancer stem cells to hedgehog pathway inhibition as a single agent, we were able to demonstrate that smoothened inhibitors are still essential in order to successfully eliminate chemoresistant pancreatic cancer stem cells if combined with other stem cell-targeting agents [9,17]. Specifically, we have previously shown for pancreatic cancer that a combination of chemotherapy and inhibitors of both mTOR and hedgehog signaling eliminates differentiated cells as well as cancer stem cells *in vitro* [9], and that this translates into long-term survival *in vivo*. Recently, Wang et al. provided an important mechanistic link for the combined inhibition of the hedgehog and mTOR pathway. Specifically, the authors demonstrate that mTOR/S6K1 signaling results in phosphorylation of Gli and subsequent expression of downstream targets. Inhibition of both pathways greatly enhanced the pro-apoptosis effect of inhibition of either inhibition alone [26]. In the present study, we now saw a virtually complete elimination of cancer stem cells for this combination therapy in a large and representative set of primary xenografts. Whereas flow cytometry using the surface markers CD133, EpCAM, and CD44 already suggested that the cancer stem cell content was strongly reduced, functional assays (e.g. sphere formation assay) validated that the cells isolated from the explanted tumors indeed were unable to form tumor spheres *in vitro*, strongly suggesting that the cancer stem cell population as the root of the disease, had been effectively targeted by the triple combination.

Even though the results using Gem-SIBI-Rapa were highly consistent and encouraging across a panel of patient-derived tumors, we did observe tumor re-growth in some mice (e.g. PDAC-185 xenografts) and, subsequently, a decrease in the survival of these xenograft-bearing animals. As this might be related to the limited bioavailability of the chemotherapeutic agent as an essential part of this combination therapy, we further advanced our treatment strategy by modifying the chemotherapy. Specifically, Vandana et al. have recently shown that modifying gemcitabine via PEGylation leads to enhanced bioavailability in the circulation as compared to native gemcitabine. Although they

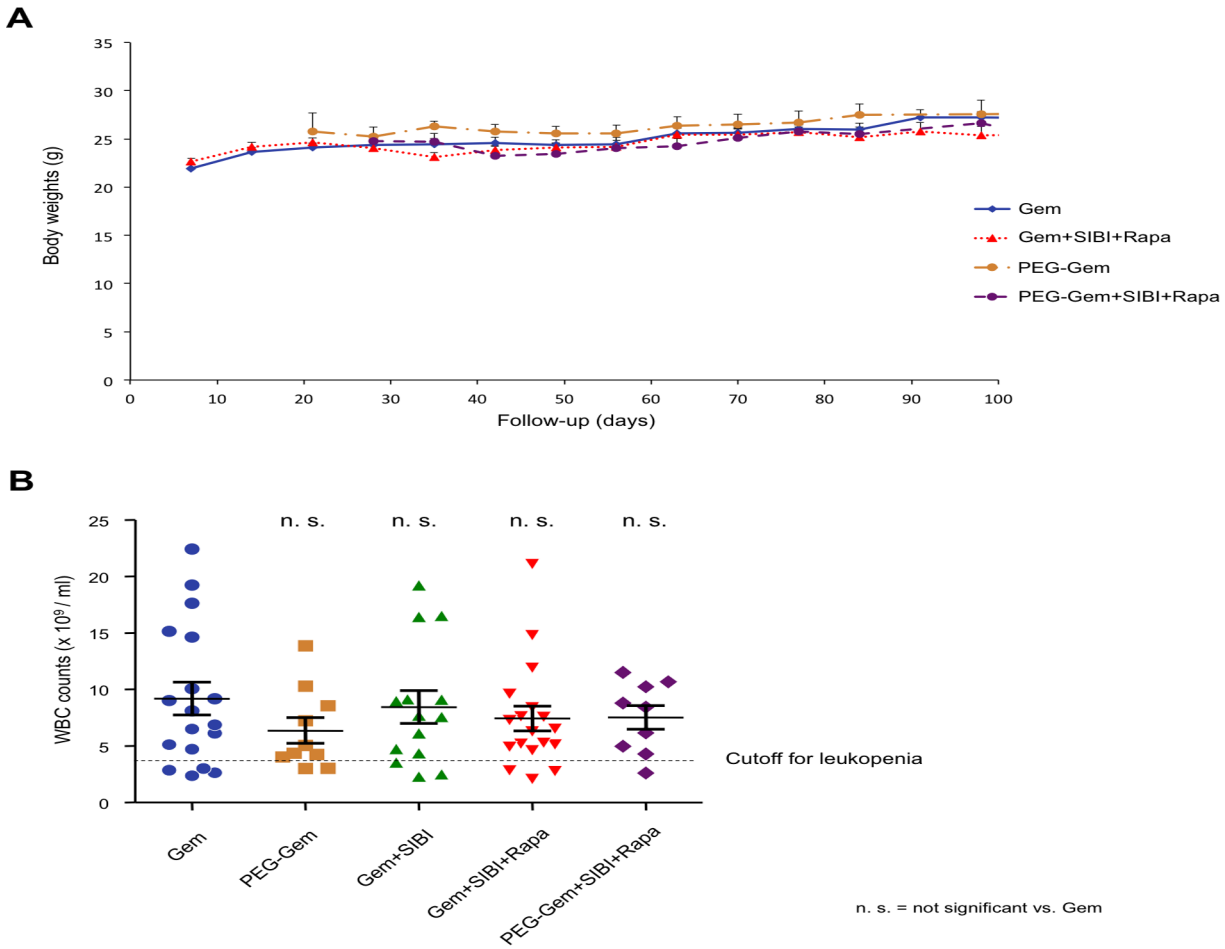

Figure 6. Assessment of in vivo biocompatibility/safety. (**A**) Body weights were recorded for all mice throughout the first 100 days of the experiment. (**B**) White blood cell counts of all mice were assessed at the end of the administration period of the triple combination.
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have also shown better *in vitro* response of established pancreatic cancer cells using PEGylated gemcitabine as compared to the regular formulation [22], we observed no significant differences between Gem and PEG-Gem *in vitro* at the level of cancer stem cell content or induction of apoptosis or cell death using xenograft-derived primary cells. While these data are not surprising as drug delivery and availability is not a critical issue *in vitro*, the results clearly emphasize the importance of utilizing primary cancer tissue for further *in vivo* evaluation of drug efficacy.

Indeed, we were then able to validate and extend this concept to the *in vivo* setting by showing that PEG-Gem treatment significantly delayed the time of tumor progression by 72 days. This enhanced treatment response is certainly impressive as it represents more than half of the study period. As expected, however, tumors ultimately progressed resulting in virtually no difference in median survival of the PEG-Gem treated mice compared to mice treated with traditional Gem alone. While these data confirm that PEGylation of Gem does indeed improve drug availability and delivery, respectively, by enhancing the circulation time and tissue penetration, as expected, PEG-Gem alone is clearly not sufficient to overcome the chemoresistance of cancer stem cells. Therefore, we next investigated the effects of replacing regular Gem with PEG-Gem in our multimodal approach for targeting pancreatic tumors, which had originally responded to Gem+SIBI+Rapa treatment, but eventually relapsed under this specific treatment regimen. Intriguingly, using the PEG-Gem+SIBI+Rapa combination we not only observed virtually complete tumor regression, but most importantly we obtained 100% survival throughout the 125d study period in this highly therapy-resistant tumor. While this observation does not exclude later re-growth as seen in PDAC-Pax22, these data are very promising and are consistent with the notion that further improving the formulation of the combined drugs is mandatory for extending *in vitro* findings to the much more complex *in vivo* setting.

The utilized new smoothened inhibitor SIBI-C1 (Siena Biotech, Italy) was also highly effective *in vivo*, as can be seen by reduced tumor growth in combination with Gem and thus significantly enhanced survival time. Furthermore, SIBI can be safely administered *in vivo*, as we saw no adverse effects on total body weight or white blood cell counts. Importantly, this is well in line with previous observations using other smoothened inhibitors [9,15]. The Gem+SIBI+Rapa combination therapy also showed no significant toxicity compared to Gem treatment alone during the course of the experiments. PEG-Gem+SIBI+Rapa combination treatment, while much more effective *in vivo*, only slightly, but non-significantly decreased white blood cell counts and had no effect on the body weight of the animals as compared to respective controls. It is important to note, however, that the healthy and relatively young mice used for this study are likely more capable of compensating for putative adverse effects on the normal stem cell

compartments during triple therapy treatment. Therefore, it will be important to further validate the safety of this treatment regimen in human patients in order to ultimately apply it to the mostly aged and moribund patients suffering from pancreatic cancer.

In conclusion, here we provide compelling evidence for the efficacy of a multimodal therapy targeting differentiated cells as well as cancer stem cells in pancreatic cancer, resulting in long-term survival in mice. Thus, these data confirm and expand previous findings from our laboratory in a very large cohort of animals with patient-derived pancreatic cancer xenografts [9,15,17]. In addition, we also offer a new and novel perspective on how to further improve current therapeutic approaches by modifying the molecular structure of the mandatory chemotherapeutic agents using PEGylation. Taken together, these findings should significantly impact the future development of new anti-pancreatic cancer therapeutics and/or treatment modalities.

Materials and Methods

Tumor Samples

After patients' informed consent had been obtained, excess tissues from resected pancreatic carcinomas was xenografted at Johns Hopkins Medical Institutions (JHMIRB: 05-04-14-02 "A Feasibility Study for Individualized Treatment of Patients with Advanced Pancreatic Cancer") and Hospital de Madrid - Centro Integral Oncológico Clara Campal (FHM.06.10 "Establishment of bank for tumors and healthy tissue in patients with cancer"), respectively, under the indicated Institutional Review Board-approved protocols [7]. Briefly, excess tumor tissues not needed for clinical diagnosis during routine Whipple resections performed by surgeons that were not involved in the present study were subsequently implanted into immunocompromised mice. All patient information was made anonymous by removal of any information, which identifies, or could lead to the identification of the patient. None of the patients had undergone neoadjuvant radiation or chemotherapy prior to resection of the tumor.

Animal Experiments

All animal experiments were conducted in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of the CNIO (Protocol PA34/2012– "Xenotransplant model for human pancreatic cancer"). Animals were housed and maintained in laminar flow cabinets under specific pathogen-free conditions. Briefly, 8 mm^3 pieces of primary, *in vivo* expanded pancreatic cancer tissue pieces were either orthotopically or subcutaneously implanted into the pancreas of 6–8 weeks old female nude mice (Harlan Europe) as described previously [7,9,27]. For each treatment group, $\geq$10 tumors were implanted. Tumor size and body weights of all animals were measured weekly. Size of the subcutaneous tumors was measured by caliper and calculated as length×width×depth. Orthotopic tumors were measured with a dedicated small-animal ultrasound system (Vevo770, Visualsonics, Toronto, Canada), and size was calculated as (length×$width^2$)/2. Survival was defined as the time point when tumors reached 1 cm^3 and mice had to be removed from the study. White blood cell counts were performed with an Abacus Junior Vet hematology analyzer (Diatron, Lenexa, Kansas).

Allocated Treatments

Gemcitabine was purchased from Lilly (Indianapolis, Indianapolis), dissolved in sterile water and administered twice a week (125 mg/kg i.p.) for 60 days. Rapa (5 mg/kg; Wyeth, Philadelphia, Pennsylvania) was orally administered via the drinking water as described previously [28]. SIBI and Rapa were administered for 21 days. SIBI-C1 (SEN826) was kindly provided by Siena Biotech S.p.A. (Siena, Italy). The characteristics of the compound are similar to that of SEN794 and SEN450, some chemical properties were ameliorated in each of the compounds. SEN450 has previously been used and characterized extensively in *in vitro* and *in vivo* tumor models of glioblastoma [20]. SIBI-C1 was dissolved in a 1:1 mixture of NaCl and polyethyleneglycol (Sigma Aldrich, St. Louis, Minnesota), and administered at 300 mg/kg by daily oral gavage. At this dose, SIBI-C1 was found to strongly inhibit the expression of the Hedgehog target genes GLI-1 and PTCH, comparable to the inhibitory effects of GDC-0449, in a subcutaneous medulloblastoma model derived from Patch+/− mice (unpublished data Siena Biotech). For *in vitro* experiments, SIBI-C1 was dissolved in DMSO and used at a concentration of 10 μM.

PEGylated gemcitabine was synthesized by Sahoo and colleagues as described previously and with modifications [22], and was administered analogous to regular gemcitabine. The PEGylated gemcitabine was synthesized by conjugating gemcitabine to HOOC-PEG-COOH in dimethylsulfoxide (DMSO), in the presence of triethylamine (TEA). Briefly, HOOC-PEG-COOH (0.1 mM) was dissolved in 2.5 ml of DMSO to which TEA (0.05 ml) was added. Further, NHS (100 mM) and EDC (400 mM) were added to the above solution and the reaction mixture was stirred for 30 min. Later, the synthesized PEG-(NHS)2 was coupled to gemcitabine. In brief, the gemcitabine (0.4 mM) was dissolved in 500 μl and added drop wise to the PEG-$(NHS)_2$ solution in the presence of 2 mM TEA (PEG-NHS/Gemcitabine/TEA molar ratio = 1:4:20). The reaction mixture was then kept on constant magnetic stirring overnight at room temperature. The reaction mixture was later subjected to dialysis using a dialysis membrane (MWCO: molecular weight cut-off = 3.5 kDa) against distilled water to remove free and unreacted gemcitabine. Subsequently, the dialyzed solution was freeze-dried using a lyophilizer (Labconco, Kansas City, Montana) at a temperature of −48°C and 0.05 mbar to obtain the powdered form of the conjugate. The characterization of the PEGylated gemcitabine was performed as described previously [22].

RNA Preparation and RT-PCR

Total RNAs from human primary pancreatic cancer cells and spheres were extracted with TRIzol kit (Life Technologies Inc.) according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis with SuperScript II reverse transcriptase (Life Technologies Inc.) and random hexamers. Quantitative real-time PCR was performed using SYBR Green PCR master mix (Qiagen), according to the manufacturer's instructions. The list of utilized primers is depicted in **Table S1 in File S2**.

Western Blot Analysis

Total protein extracts were obtained using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) supplemented with phosphatase and protease inhibitors. Pellets were incubated during 1 h in lysis buffer at 4°C, and centrifuged at 13,000 rpm during 10 min at 4°C. Total protein concentration was measured with BCA Protein Assay kit (Pierce) and 25–100 mg protein were separated by SDS/PAGE and transferred to PVDF membranes. Upon antibody incubation, membranes were visualized by enhanced chemoluminescence (Amersham). GAPDH was used as a loading control. A complete list of used antibodies is included in **Table S2 in File S2**.

Flow Cytometry

To characterize pancreatic cancer stem cells, the following antibodies were used: anti-CD133/1-APC (clone AC133 Miltenyi Biotech, Bergisch Gladbach, Germany), anti-CD44-PE anti-EpCAM FITC (both Becton Dickinson, Heidelberg, Germany) or appropriate isotype-matched control antibodies. Samples were analyzed by flow cytometry, using a FACSCanto II (BD), and data were analyzed with FloJo 9.4.4 (Treestar, Ashland, Oregon). Apoptosis and cell death analyses were performed using DAPI and an Annexin V fluorescein isothiocyanate (FITC) staining kit (BD).

Histology

Formalin-fixed, paraffin-embedded tumor sections were stained with a CK19 antibody (1:500, Dako, Carpinteria, CA), and then visualized with a rabbit anti-mouse and anti-rabbit HRP-conjugated antibody (both Epitomics). Nuclear counterstaining was performed using Hematoxylin. The stroma quantification was performed by two independent investigators, one an experienced pathologist (E.G.).

Cell Culture

For *in vitro* studies, tumors were enzymatically digested with collagenase and pancreatic cancer adherent cell and sphere cultures were generated and expanded as previously described [17,18]. Five thousand cells per milliliter were seeded in ultra-low attachment plates (Corning B.V., Schiphol-Rijk, Netherlands) and monitored for sphere formation capacity over the course of 14 days. Spheres were defined as 3-dimensional multicellular structures of approximately 40 µm or larger. For in vitro treatment, 10^5 cells per well were seeded in 6-well plates, treated with either standard gemcitabine or PEG-Gem at a concentration of 100 ng/mL after 24 h, and analyzed on day 3 by flow cytometry to detect apoptosis and cancer stem cell content.

Statistical Analysis

Results for continuous variables are expressed as means ± standard error of the mean (SEM) unless stated otherwise. Overall comparison of continuous variables was performed with the Kruskal-Wallis test followed by post hoc pairwise comparison using the Mann-Whitney U test. Survival was compared using a Log Rank test. P values <0.05 were considered statistically significant. All analyses were performed with SPSS 19 (SPSS Inc., Chicago, IL).

Supporting Information

File S1 Figure S1A: Effect of SIBI-C1 on Hedgehog pathway gene expression. Fold increase mRNA expression levels of SHH and GLI2 in gemcitabine resistant primary cancer cells (PDAC-265 left panel, PDAC-354 right panel). **Figure S1B: Effect of combination therapy on tumor composition.** Representative histological pictures showing stroma content in the respective treatment groups in gemcitabine resistant subcutaneously implanted tumors (PDAC-185, upper panel), (354, lower panel).
(TIF)

File S2 Table S1: Utilized qRT-PCR primers. Table S2: Utilized antibodies.
(TIF)

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Author Contributions

Conceived and designed the experiments: PCH SMT SKS CH. Performed the experiments: PCH SMT BS A. Balic. Analyzed the data: PCH SMT PT A. Balic CH BS EG. Contributed reagents/materials/analysis tools: SAH MH PT A. Bakker MV SKS. Wrote the paper: PCH BS CH.

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DISCUSSION

According to the cancer progression model postulated by Fearon and Vogelstein in 1990, at least 4-5 genetic events are required for the progression from normal epithelium to carcinoma in colorectal cancer (Fearon and Vogelstein, 1990), and a similar number of key genetic alterations seem to be necessary for the development of pancreatic adenocarcinoma. However, it is currently unclear, in which cell these genetic alterations accumulate, leading to the cell's malignant transformation. Intensive efforts are being undertaken to identify this "cell-of-origin" using various genetically engineered mouse models encoding the key genetic alterations under different cell-specific promoters for pancreatic (Guerra et al., 2007, Hingorani et al., 2005, Kopp et al., 2012) and colorectal cancers (Fearon, 2011, Fearon and Vogelstein, 1990). Even though their precise origin remains yet to be elucidated, the existence of CSC or tumour-initiating cells has now been conclusively proven in most epithelial tumours, and more and more evidence is arising that these CSC are indeed clinically relevant due to their pronounced resistance to standard therapies (Hermann et al., 2007, Gallmeier et al., 2011, Mueller et al., 2009, Todaro et al., 2007, Bao et al., 2006). The resistance of cancer stem cells to standard treatment in combination with their capability to recapitulate a tumour in all its cellular heterogeneity (one of the defining features of cancer stem cells) provides a reasonable explanation for the clinical observation that tumours will relapse after initially successful treatment (**Figure 5a**). Thus, targeting cancer stem cells in addition to differentiated tumour cells will be of paramount importance in order to successfully treat patients with cancer. Applying this idea clinically would mean specifically targeting CSCs, while at the same time maintaining established therapeutic protocols of standard therapy such as chemotherapy or radiation (**Figure 5b**).

Different approaches are conceivable to successfully target CSCs: either making them accessible to standard treatment (e.g. driving normally quiescent CSCs into a proliferative state, thus making them more susceptible for chemotherapy), or killing these cells directly by interfering with key signaling pathways. Here we provide evidence for both approaches, showing three new therapeutic options to treat pancreatic and colorectal cancer stem cells.

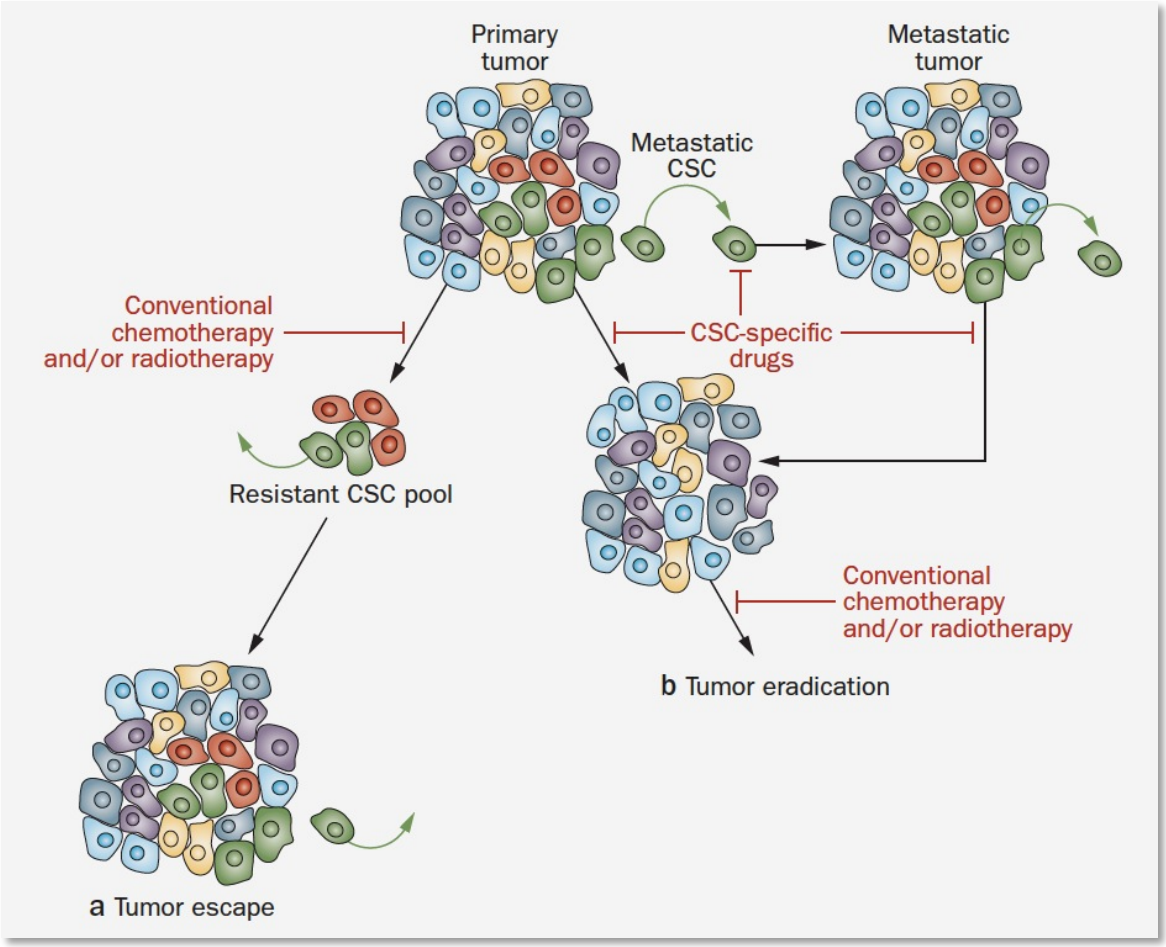

Figure 5: Combination therapy to eliminate cancer stem cells as well as differentiated tumour cells. CSCs (red) and their metastatic subclones (green) escape conventional therapy and re-capitulate tumors and/or metastases, unless anti-CSC therapies are used simultaneously. From: (Sergeant et al., 2009)

In the course of this thesis and the projects included therein, we have validated the concept of a multimodal therapy for comprehensively targeting the diverse cell compartments in pancreatic and colorectal cancer using a large set of more than 200 subcutaneous and orthotopic whole-tissue primary tumour xenografts, making this one of the largest investigations in the cancer stem cell field.

Colorectal Cancer Stem Cells and Inhibition of ATR

We demonstrate here that inhibition of ATR function depletes the tumourigenic CD133+ fraction of established colon cancer cell lines as well as xenograft-derived

primary colon cancer cells. This effect translated into a markedly reduced tumourigenicity of the remaining cells, as shown by an impaired sphere formation capacity *in vitro* (Ricci-Vitiani et al., 2007, Todaro et al., 2007) as well as a strongly reduced capability to form tumours *in vivo*. Consistent with previous reports describing amplified checkpoint activation and increased DNA repair to be distinct features of some CSC (Bao et al., 2006, Eyler and Rich, 2008), CD133+ cells displayed a stronger activation of the ATR- dependent DDR on treatment with interstrand-crosslinking (ICL) - agents than did CD133- cells, as evidenced by a more pronounced increase in phosphorylation of ATR's major effector kinase CHK1. Importantly, the depletion of CD133+ cells was enhanced on subsequent treatment with ICL-agents, suggesting that inhibition of ATR might reverse the chemoresistance of CSC toward ICL-agents in the clinical setting and could thus serve as a novel therapeutic strategy for patients suffering from colon cancer.

As the PIK kinases ATM, ATR, MTOR, and DNA-PK all play pivotal roles in cell cycle checkpoint functions and all except DNA-PK are effectively inhibited by caffeine (Sarkaria et al., 1999), caffeine was used as a screening approach to modulate check-point function in colon cancer cells. Caffeine treatment virtually abolished the CD133+ cell fraction and was accompanied by a decreased *in vitro* and *in vivo* tumourigenicity of the remaining cell population, providing functional evidence for a successful targeting of the tumour-initiating CSC fraction. Depletion of CD133+ cells was observable as early as 5 days after caffeine administration and further enhanced after longer exposure, indicating that prolonged treatment was required for the complete exhaustion of the CD133+ subpopulation, first through the elimination of the rapid cycling cell fraction and consecutively through activation of a slow cycling or even quiescent fraction. Consistently, caffeine increased the proliferating fraction of CD133+ cells in our experiments.

A panel of small molecule inhibitors was applied to dissect the contributions of the different PIK kinases on the caffeine-induced preferential depletion of CD133+ cells. As no specific ATR-inhibitors are currently available, several potent, but in comparison with the highly specific ATM inhibitors, less-specific inhibitors of CHK1 as the major effector kinase of ATR were used as surrogates for ATR inhibition. In contrast to the ATM inhibitor KU-55399 and the MTOR inhibitor RAD001, only CHK1 inhibitors mimicked the effects of caffeine. As CHK1 activity itself is only marginally suppressed by caffeine (Sarkaria et al., 1999), whereas the upstream PIK kinase ATR is potently

inhibited, these data suggested that the caffeine- induced depletion of CD133+ cells was mediated through direct inhibition of ATR followed by indirect inhibition of its main effector kinase CHK1. Importantly, caffeine exerted its detrimental effects on CD133+ cells already after short-term treatment, whereas the effects of CHK1 inhibitors were observable only after long-term treatment. To exclude different pharmacokinetic properties of the used agents as the underlying reason for the observed differences, our findings were corroborated by a set of siRNA experiments. Consistently, knockdown of CHK1 protein expression over 264 hours did not lead to comparable detrimental effects on the CD133+ cell population as did ATR protein depletion. Furthermore, we analyzed an isogenic FA knockout model (Gallmeier et al., 2006, Gallmeier and Kern, 2007), as ATR had been linked to the FA DNA-repair pathway (Andreassen et al., 2004), but found no evidence for an impact of FA pathway abrogation on the depletion of CD133+ colon cancer cells. Together, these data suggest that besides CHK1 as the major effector kinase of ATR, other ATR-dependent, but FA-independent pathways are operative in this setting.

The complete disruption of the ATR gene is a lethal event in human somatic cells (Cortez et al., 2001) and no applicable cellular model presently exists to investigate the null state of the ATR gene. However, the hypomorphic ATR-inactivating splicing mutation $2101^{A\rightarrow G}$, naturally found in Seckel syndrome patients (O'Driscoll et al., 2003), causes subtotal depletion of ATR protein without gross effects on cancer cell growth or viability (Hurley et al., 2007, Gallmeier and Kern, 2007). Therefore, cancer cells homozygously harboring this mutation (ATRs/s cells) were used as a highly specific tool to model ATR inhibition in tumours. ATRs/s cells were virtually depleted of CD133+ cells as compared with parental ATR+/+ cells. Consistently, ATRs/s cells were impaired in sphere formation capacity and unable to form tumours *in vivo*. ATRs/s cells did not show significant differences in proliferation rates as compared with their ATR+/+ counterparts, excluding that their loss of tumourigenicity was attributable to a hypothetical cell cycle arrest. It should be noted that a limitation of our genetic ATR model is that confounding artifacts due to clonal variability cannot definitively be excluded (Gallmeier and Kern, 2007). Therefore, our data require cautious interpretation, especially when considering the CD133 expression status of the originally derived ATRs/s cell clones. As can be derived from our initial experiments, tumourigenicity was mainly restricted to the CD133+ cell fraction of DLD1 colon cancer cells, which constituted only about 5% of the unselected DLD1 cell population. Thus, the engineered ATRs/s cells were more likely

originally derived from a CD133- cell clone, which according to our data, would be expected not to be capable of regenerating tumourigenic CD133+ cells, at least in our short- term experimental setting (14 days). On the other hand, it remains a controversial issue whether non-CSC or a subpopulation of them might be able to regenerate CSC in the long run, or correspondingly, whether CD133- ATR+/+ cells might at some point regenerate CD133+ cells (Chen et al., 2010). Taken together, our data demonstrate that ATR-deficient CD133- cancer cells retain a non-tumourigenic phenotype for at least several months during cell culture.

To exclude potential artifacts due to clonal variability, we employed a third model of ATR function, using RNA-interference through repetitive application of ATR siRNA, which facilitated the continuous depletion of ATR protein in unselected colon cancer cell populations. Similar to the results obtained in the genetic model, we observed a time-dependent decrease of the CD133+ cell fraction in ATR siRNA-treated cells along with a concomitant reduction of the *in vivo* tumourigenicity of the remaining cell population, strongly indicative of a successful targeting of the tumour-initiating stem cell fraction. In contrast to ATRs/s or caffeine-treated cells, however, ATR siRNA-treated cells did not exhibit a complete abrogation of *in vivo* tumour formation in all animals. This could most likely be ascribed to an inevitable methodological shortcoming of experiments applying siRNA technology, that is, the incomplete targeting on the cellular level, generally leaving a remaining sub- population of not efficiently siRNA-transfectable cells (including CD133+ cells), unaffected. Accordingly, traceable amounts of ATR protein were still detectable in the ATR siRNA-treated cell population after 384 hours of repetitive siRNA-application. As a consequence, the decreased, but maybe not absent, tumourigenic cell fraction on ATR siRNA treatment would be expected to lead to a significantly decreased, but not absent, tumour take rate, as observed in our experiments. Interestingly, in those rare instances, in which tumours were generated by ATR siRNA-treated cell populations, these tumours were clearly diminutive as compared with those observed in control mice. This could be explained by the decreased fraction of tumourigenic CD133+ cells in the ATR siRNA-treated population, as a smaller fraction of tumour-initiating cells could perceivably also account for a decreased tumour size in those rare instances of successful tumour formation. Indeed, a very rare occurrence of CD133+ cells was observable in tumours that originated from ATR siRNA-treated cells. Another explanation, consistent with the observed sparse proliferation activity of the diminutive tumours *in vivo*, would be that these tumours did not arise from the successfully

eliminated tumour-initiating cell fraction but rather from an untargeted subset of short-lived transient amplifying cells, which only divide a finite number of times until they become terminally differentiated and finally undergo senescence (Barker et al., 2009).

A preferential activation of the DDR, comprising both amplified checkpoint activation and increased DNA-repair, has previously been proposed as a likely mechanism of CSC drug- resistance (Bao et al., 2006, Eyler and Rich, 2008) and could also explain the increased sensitivity of CD133+ colon cancer cells toward ATR inhibition; ATR is a central regulator of the replication checkpoint, which blocks cell cycle progression on detection of endogenous or exogenously induced SRF. In this process, ATR stabilizes SRF via its main effector kinase CHK1 and prevents the inappropriate processing of DNA (Paulsen and Cimprich, 2007). Accordingly, cells harboring a complete disruption of the ATR gene display increased chromo- some breaks even in the absence of exogenous replication stress, most likely induced through SRF occurring during nor- mal cellular proliferation, and are not viable over extended periods of time (Cortez et al., 2001). The significantly stronger up-regulation of CHK1 phosphorylation in the CD133+ as compared with the CD133- cell fraction on treatment with SRF-inducing ICL- agents in our experiments thus supports a preferential activation of the ATR-dependent DDR also in colon CSC.

Consistently, treatment with SRF-inducing ICL-agents accelerated the depletion of CD133+ cells on ATR inhibition, further supporting that the detrimental effects of caffeine specifically on CD133+ cells were attributable to the particularly reduced capability of these cells to repair, endogenously or exogenously inflicted, SRF when ATR function was impaired. It is tempting to speculate that the impaired DNA repair capability of CD133+ cells in response to ATR inhibition could be ascribed structurally to differences in chromatin compaction between CD133+ and CD133- cells. Overall, chromatin accessibility, a dynamic process largely mediated by chromatin compaction, represents an innate property of stem cells, which is lost during differentiation (Meshorer et al., 2006). The degree of chromatin compaction, on the other hand, determines at least in part the extent of DNA damage, the feasibility of DNA repair (Cohn and D'Andrea, 2008), and the strength of the DDR (Murga et al., 2007) and could thus explain a particularly strong dependence of CSC on an intact DDR.

On confrontation with DNA damage, the DDR mediates whether cells undergo a replication arrest to allow DNA repair, bypass the DNA damage and continue to replicate DNA, or eventually, undergo apoptosis (Harper and Elledge, 2007). We found that after

caffeine treatment, apoptotic cell death did not occur immediately in CD133+ cells, but progressively increased with cumulative BrdU incorporation, excluding cytotoxicity as the sole source of the caffeine-induced effects. Notably, a small fraction of AnnexinV+ CD133+ cells was detectable up to 168 hours after treatment initiation, likely representing the CSC fraction progressively recruited to enter an active cell cycle. Consistently, after an initial caffeine-induced increase of CD133+ AnnexinV+ cells, their amount subsequently declined, paralleling the decline of total CD133+ cell numbers. Thus, the caffeine-induced depletion of CD133+ cells was at least in part attributable to proliferation-dependent induction of apoptosis. As apoptosis was triggered by caspase 8 and reinforced by a mitochondrial amplification loop involving the recruitment of caspase 9, sensitization to extrinsic receptor-mediated apoptosis might provide another tool for the specific depletion of the CSC fraction in colon cancer.

Using three independent model systems, that is, pharmacological ATR inhibition, genetic inactivation of the ATR gene, and RNA interference-mediated ATR protein depletion, we found that inhibition of ATR function depleted the tumourigenic CD133+ cell fraction of established human colon cancer cell lines as well as xenograft-derived primary colon cancer cells. This effect was attributable at least in part to apoptosis, accelerated on co-treatment with common chemotherapeutics that generate SRF, and accompanied by a drastically decreased *in vitro* and *in vivo* tumourigenicity of the remaining cells. Mechanistically, the preferential depletion of tumourigenic CD133+ cells was attributable to the preferential activation of the ATR-dependent DDR in these cells. Our study thus illustrates a novel approach to selectively eliminate the tumourigenic cell population in colon cancer. As the caffeine blood levels required for inhibiting ATR function cannot be achieved *in vivo* due to the narrow therapeutic window and the pronounced cardiovascular side effects of caffeine and its derivatives, our study provides a strong rationale for the pharmaceutical development of specific ATR inhibitors as a potentially powerful approach to eliminate CSC in colorectal cancer (Nghiem et al., 2001, Collis et al., 2003, Wilsker and Bunz, 2007).

Pancreatic Cancer Stem Cells and Inhibition of Nodal / Activin

Patients with pancreatic ductal adenocarcinoma are still suffering from a devastating prognosis, which can be at least partially rationalized by the observation that the standard chemotherapeutic agent gemcitabine is not capable of eliminating CSCs.

Indeed, gemcitabine rather leads to a relative increase in the number of CSCs, indicating a preferential targeting of more differentiated and rapidly proliferating cancer cells. The restricted elimination of the more differentiated cancer cells, even if associated with significant tumour size reduction, will not lead to the eradication of the tumourigenic potential of the tumour, as that is restricted to the CSC population, a population that is highly resistant (Hermann et al., 2007, Mueller et al., 2009, Gallmeier et al., 2011, Bao et al., 2006), survives the standard therapy, and maintains the ability to re-populate a tumour in all its heterogeneity. Therefore we evaluated the effects of combination therapies targeting both differentiated cancer cells and CSCs on a panel of pancreatic tumours, which were selected based on their previously described differential response to gemcitabine treatment (Jimeno et al., 2009). Using these tumours, here we demonstrate that the Nodal/Activin pathway is essential for the self-renewal capacity and stemness properties of pancreatic CSCs. Nodal/Activin is strongly expressed in pancreatic CSCs, but is also expressed by pancreatic stellate cells, which are abundantly present in the stroma surrounding pancreatic cancer cells and may serve as a CSC niche.

In a large set of primary cells and (fresh) primary patient tissues, we then showed that the CSC compartment is severely affected by inhibition of this pathway by making use of three different approaches: first, by using a small molecule inhibitor (SB431542) targeting the Nodal/Activin receptor Alk4; second, by using recombinant Lefty as the specific endogenous Nodal inhibitor; and third, by genetic knockdown of Nodal, Alk4, and Smad4 using shRNA technology. Our findings are in line with earlier observations that have identified other developmental pathways such as mTOR, hedgehog, Notch, and BMP for targeting CSCs (Bar et al., 2007, Li et al., 2007, Mueller et al., 2009, Piccirillo et al., 2006), although their targeting may be of limited clinical use for at least some of them due to normal stem-cell-related side effects. Intriguingly, an important feature of the herein described Nodal/Activin pathway is its complete lack of activity in normal pancreas and other adult tissue (Topczewska et al., 2006), spurring the hope for little to no side effects because normal stem cells will most likely be spared.

Nodal and Activin are involved in developmental biology by perpetuating the undifferentiated state of ESCs (Vallier et al., 2005, Xiao et al., 2006). While the expression of Activin and the Nodal co-receptor Cripto-1 have previously been demonstrated in pancreatic cancers (Friess et al., 1994, Kleeff et al., 1998), we here provide evidence that Nodal, the second ligand of the Alk4/7 receptor, is expressed in

this malignancy, but not in normal pancreas. Most importantly, Nodal is capable of strongly propagating the tumourigenic CSC subpopulation as demonstrated by its pharmacological inhibition using the extra- cellular Nodal antagonist Lefty and shRNA technology, whereas Activin was less drastically enriched in pancreatic CSCs and showed limited effects on their self-renewal capacity in some tumours. These data are in line with previous reports showing that Nodal is crucial for tumourigenicity in melanoma and breast cancer cells, with an embryonic microenvironment reducing tumourigenic activity and inducing the expression of epithelial markers by the secretion of Lefty (Postovit et al., 2008, Topczewska et al., 2006).

On the other hand, Activin reportedly contributes to an invasive phenotype in esophageal carcinoma, another epithelial malignancy (Yoshinaga et al., 2004, Yoshinaga et al., 2008). In a previous report on the dynamic regulation of the invasive phenotype of breast cancer cell lines, the interconversion from noninvasive epithelial-like CD44+CD24+ cells to invasive mesenchymal CD44+CD24- progeny was also found to be Nodal/Activin dependent (Meyer et al., 2009). Consistently, we now provide evidence that Activin also promotes invasion of pancreatic CSCs as does Nodal. These data have important implications because they indicate that therapeutic strategies should not focus on either Nodal or Activin, but rather focus on Alk4/7 as their common receptor. Indeed, a comprehensive set of experiments proves that targeting this pathway by blocking the Alk4/7 receptor using the small molecule inhibitor SB431542 and shRNA technology has a strong impact on both the CD133+ fraction that is enriched for CSCs and sphere formation capacity.

Next, we identified human pancreatic stellate cells (PSCs) as an important component of the stroma that also strongly expresses Nodal/Activin. Conditioned medium from PSCs promoted self-renewal and invasive- ness of pancreatic CSCs. PSCs, which reside in exocrine areas of the pancreas, are myofibroblast-like cells known to be activated upon insult. These cells are analogous to hepatic stellate cells, with which they share 99% identity at the transcriptome level (Omary et al., 2007). PSCs are important mediators in the pancreatic response to injury because they migrate to the damaged location and promote cell proliferation, migration, and assembly (Shimizu, 2008). Therefore, because our data suggest that PSCs may represent an *in vivo* niche for CSCs, targeting these interactions could be of pivotal importance for the development of more effective therapies for pancreatic cancer. While targeting Alk4/7 as the common receptor for Nodal/Activin should abrogate autocrine and paracrine

signaling, directly eliminating this paracrine source for Nodal/Activin may provide additional therapeutic benefits. Intriguingly, this can be achieved by targeting the hedgehog pathway as a crucial signaling component for PSCs (Bailey et al., 2008, Shinozaki et al., 2008) and may account, at least in part, for the striking therapeutic effects generated by the addition of a smoothened inhibitor to our armamentarium for treating primary pancreatic cancer tissue in our studies (**Figure 6**).

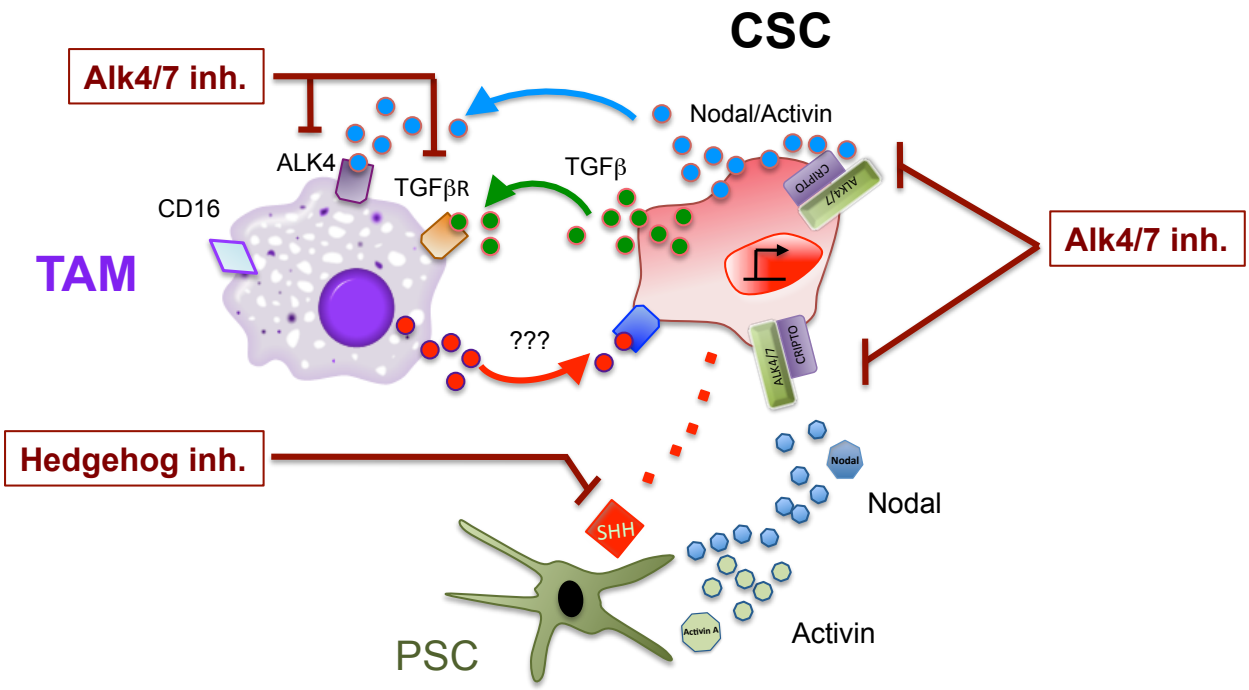

Figure 6: Combination therapy targeting cancer stem cells (CSC). Direct targeting of CSC as well as interfering with crucial interactions between cancer stem cells and stromal cells (pancreatic stellate cells (PSC) and tumour-associated macrophages (TAM)) by inhibiting the hedgehog pathway or the Nodal/Activin pathway via Alk4/7.
Image courtesy of C. Heeschen

However, translating our findings into the *in vivo* setting was not only challenged by alternative sources for Nodal/Activin, but also by the fact that the Nodal/Activin small molecule inhibitor SB431542 as a single therapy was not sufficient to irreversibly eliminate the cells' ability to form tumours *in vivo*. This lack of *in vivo* translation of the apparently encouraging *in vitro* effects could be explained by the enhanced plasticity of pancreatic cancer cells. Indeed, after withdrawal of SB431542 and continued culture of the cells, a drastic rebound of the CD133+ population was also observed *in vitro*, which

retrospectively rationalizes the still-preserved *in vivo* tumourigenicity of the cells. However, the rebound of CD133+ CSCs upon withdrawal was prevented by addition of gemcitabine to the treatment regimen. Further mechanistic studies revealed that SB431542 alone (reversibly) drives CSCs into a more differentiated state, as evidenced by loss of CD133, but cells still retain the ability to revert to the CSC phenotype. Intriguingly, although gemcitabine alone led to a relative enrichment of CSCs, the combination of SB431542 and gemcitabine resulted in their irreversible and complete elimination. Indeed, *in vitro* combination therapy resulted in complete abrogation of the *in vivo* tumourigenic potential of the remaining cells.

This chemosensitizing effect of SB431542 should be of great therapeutic value for patients with pancreatic cancer and was therefore further evaluated *in vivo*. However, testing this treatment regimen in mouse models of pancreatic cancer came with another caveat. Our first *in vivo* experiments in established pancreatic cancer, which were based on the orthotopic implantation of isolated pancreatic cancer cells, confirmed the *in vitro* data by illustrating robust therapeutic efficacy and 100% survival at 100 day follow-up for SB431542 plus gemcitabine. Surprisingly, however, when we then moved to a preclinical model using xenografted primary human pancreatic cancer tissue, tumour development remained virtually unaffected by this combination. It is important to note that xenografted pancreatic cancer tissues contain large amounts of stroma whereas implantation of cancer cells regularly lacks this important feature. Tumour-associated stroma does not only provide an additional source for Nodal/Activin as described above, but is also capable of modulating tumour vascularization, which could interfere with drug delivery to cancer (stem) cells. Indeed, impaired drug delivery has already been demonstrated for pancreatic cancer in a recent landmark study using a genetically engineered mouse model (Olive et al., 2009).

Therefore, breaching the ‘‘stroma fortress’’ of pancreatic cancer represents an important challenge for drug delivery in general (Neesse et al., 2010) and CSC-targeted therapies in particular because these cells have been proposed to preferably reside in hypoxic niches (Borovski et al., 2011, Heddleston et al., 2009). Intriguingly, when we co-administered the hedgehog pathway inhibitor CUR199691 (Mueller et al., 2009) to deplete the stromal compartment together with SB431542, we observed a 10-fold increase in drug delivery into the tumour tissue. The addition of gemcitabine then translated into rapid disease stabilization, and none of the mice required sacrificing during the 100 day study period. Failure to completely eradicate the remaining small

tumours can be rationalized by the lack of response of non-proliferating tumour cells to gemcitabine. Most importantly, however, these small lesions no longer contain CSCs; cells isolated from these remnant tumours did not form spheres anymore. In contrast, all mice treated with gemcitabine alone had to be sacrificed within 100 days due to excessive tumour growth. Cells isolated from these tumours bear strong sphere forming capacity. Therefore, our data demonstrate the successful combination of stroma- and CSC-targeting strategies for effectively treating pancreatic cancer in most relevant preclinical models.

Canonical downstream signaling of Alk4/7 is mediated by Smad2/3, as well as by the co-Smad Smad4, which is shared by all TGF-b family members. Importantly, about 50% of patients with pancreatic cancer bear inactivating mutations or deletions of the Smad4 gene, which could result in dysfunction of the pathway (Schneider and Schmid, 2003). While non-canonical TGF-b family signaling pathways have been described and may account for the enhanced TGF-b1-induced invasiveness of pancreatic CSCs (Zhang, 2009), we found that Smad4 knock- down in previously Smad4-competent cells resulted in reduced *in vivo* tumourigenicity, most likely via inhibition of Nodal/Activin signaling, because these cells no longer responded to the Alk4/7 inhibitor SB431542. Therefore, because Smad4 seems indispensable for the Nodal/Activin signaling cascade, tumours carrying functionally relevant Smad4 mutations or deletions may not respond to a Nodal/Activin-targeting therapy. Importantly, however, not all Smad4 mutations actually result in dysfunctional Smad4; we have identified several tumours bearing Smad4 mutations that still demonstrate a functional Smad2/3 cascade, including a subsequent translocation of pSmad2 into the nucleus, and that respond to this triple therapy. Future studies will have to address the question of which patients will most likely respond to this treatment modality and how best to identify them.

Pancreatic Cancer and Inhibition of Hedgehog and mTOR

Based on the observation that pancreatic cancer stem cells are highly resistant to standard therapies (Hermann et al., 2007), we demonstrated in 2009 that this resistance can be overcome, using a combined inhibition of Sonic Hedgehog (SHH) and the mTOR pathway, together with gemcitabine as a chemotherapeutic agent (Mueller et al., 2009).

While this study was the first systematic investigation into the effects of combination therapy on pancreatic cancer stem cells, only a comparatively small number of patient-derived tissue xenografts was used for this study. Therefore, in the aforementioned study (Hermann et al., 2013) we extend the findings, using a large panel of primary pancreatic cancer xenografts with distinct response profiles to gemcitabine (Jimeno et al., 2009), and different mutational profiles (Jones et al., 2008). Using almost 200 primary xenografts, this study is one of the largest investigations of combination therapies in the cancer stem cells. Furthermore, we used SIBI-C1 (Siena Biotech, Siena, Italy) as a new inhibitor of Smoothened, and thus of the hedgehog pathway.

At the histological level, pancreatic cancer is characterized by very dense stroma and poor vascularization. Olive et al. showed in a genetically engineered mouse model of pancreatic cancer that the stroma is strongly dependent on hedgehog signalling, and inhibition of the hedgehog pathway with smoothened inhibitors leads to ‘‘preferential’’ killing of stromal cells and increased vessel density (Olive et al., 2009), thus making tumour cells more accessible to therapeutic intervention. While these observations were obtained in a mouse model of pancreatic cancer, we have more recently shown that also in patient-derived whole-tissue xenografts co-treatment with a smoothened inhibitor significantly increases drug delivery (Lonardo et al., 2011) and markedly reduces tumour-associated stroma formation. Importantly, we now appreciate that the stroma not only hampers drug delivery (Olive et al., 2009), but also provides a supportive niche for cancer stem cells promoting their self-renewal capacity and invasiveness (Lonardo et al., 2012). Thus, elimination or abrogation of the stroma does significantly improve treatment regimens by distinct mechanisms, but is only capable of eliminating cancer stem cells if combined with Gem. Interestingly, we have shown previously that interfering with the hedgehog pathway via inhibition of Smoothened is not sufficient to deplete cancer stem cells (Mueller et al., 2009), but will only lead to CSC eradication in combination with mTOR inhibition and gemcitabine chemotherapy. However, these *in vitro* data have to be interpreted carefully, since Lauth and colleagues have shown recently that in the context of an activating K-Ras mutation, the Gli family of hedgehog target genes is up-regulated independently of Smoothened (Lauth et al., 2010). This is particularly interesting, considering that mTOR activity, similarly to K-Ras, activates Gli1 independently of Smoothened (Wang et al., 2012), explaining the need for additional mTOR inhibition observed in our studies. Thus, using Gli-inhibitors may not only improve stroma depletion, but may also more successfully target cancer stem cells.

However, despite the rather modest response of cancer stem cells to hedgehog pathway inhibition as a single agent, we were able to demonstrate that smoothened inhibitors are still essential in order to successfully eliminate chemoresistant pancreatic cancer stem cells if combined with other stem cell-targeting agents (Mueller et al., 2009, Lonardo et al., 2011). Specifically, we have previously shown for pancreatic cancer that a combination of chemotherapy and inhibitors of both mTOR and hedgehog signaling eliminates differentiated cells as well as cancer stem cells *in vitro* (Mueller et al., 2009), and that this translates into long-term survival *in vivo*. Recently, Wang et al. provided an important mechanistic link for the combined inhibition of the hedgehog and mTOR pathway. Specifically, the authors demonstrate that mTOR/S6K1 signaling results in phosphorylation of Gli and subsequent expression of downstream targets. Inhibition of both pathways greatly enhanced the pro-apoptotic effect of inhibition of either inhibition alone (Wang et al., 2012). In the presented study, we now saw a virtually complete elimination of cancer stem cells for this combination therapy in a large and representative set of primary xenografts. Whereas flow cytometry using the surface markers CD133, EpCAM, and CD44 already suggested that the cancer stem cell content was strongly reduced, functional assays (e.g. sphere formation assay) validated that the cells isolated from the explanted tumours indeed were unable to form tumour spheres *in vitro*, strongly suggesting that the cancer stem cell population as the root of the disease, had been effectively targeted by the triple combination.

Even though the results using Gem+SIBI+Rapa were highly consistent and encouraging across a panel of patient-derived tumours, we did observe tumour re-growth in some mice (e.g. PDAC-185 xenografts) and, subsequently, a decrease in the survival of these xenograft-bearing animals. As this might be related to the limited bioavailability of the chemotherapeutic agent as an essential part of this combination therapy, we further advanced our treatment strategy by modifying the chemotherapy. Specifically, Vandana et al. have recently shown that modifying gemcitabine via PEGylation leads to enhanced bioavailability in the circulation as compared to native gemcitabine. Although they have also shown better *in vitro* response of established pancreatic cancer cells using PEGylated gemcitabine as compared to the regular formulation (Vandana and Sahoo, 2010), we observed no significant differences between Gem and PEG-Gem *in vitro* at the level of cancer stem cell content or induction of apoptosis or cell death using xenograft-derived primary cells. While these data are not surprising as drug delivery and availability is not a critical issue *in vitro*, the results

clearly emphasize the importance of utilizing primary cancer tissue for further *in vivo* evaluation of drug efficacy.

Similar observations have been made recently regarding abraxane, an albumin-bound paclitaxel used as chemotherapeutic agent (Von Hoff et al., 2011). Pre-clinical studies with abraxane have demonstrated that binding paclitaxel to albumin facilitates drug delivery *in vivo*, resulting in a significantly increased intra-tumoural concentration of gemcitabine in mice treated with nab-paclitaxel versus those receiving gemcitabine alone. Interestingly, abraxane as single agent as well as together with gemcitabine depleted the typically dense and almost avascular tumour stroma in PDAC, markedly enhancing drug delivery into the tumor. Whereas this treatment resulted in markedly increased patient survival in a clinical phase I/II study (Von Hoff et al., 2011), increasing survival time from 8.1 to 17.8 months, all patients eventually succumbed to the disease. Thus, the results from this study are certainly very promising from a clinical perspective and support the idea of modifying chemotherapeutic agents to improve their efficacy. However, with all patients eventually dying, it becomes apparent that even stromal depletion and improved delivery of chemotherapeutics into the tumour, populations of chemoresistant cancer stem cells have survived the chemotherapy and given rise to a relapsing tumour, eventually killing the patient. This further supports the notion that multimodal therapies will be necessary for successful treatment.

Indeed, we then validated and extended this concept to the *in vivo* setting by showing that PEG-Gem treatment significantly delayed the time of tumour progression by 72 days. This enhanced treatment response is certainly impressive as it represents more than half of the study period. As expected, however, tumours ultimately progressed resulting in virtually no difference in median survival of the PEG-Gem treated mice compared to mice treated with traditional Gem alone. While these data confirm that PEGylation of Gem does indeed improve drug availability and delivery, respectively, by enhancing the circulation time and tissue penetration, as expected, PEG-Gem alone is clearly not sufficient to overcome the chemoresistance of cancer stem cells. Therefore, we next investigated the effects of replacing regular Gem with PEG-Gem in our multimodal approach for targeting pancreatic tumours, which had originally responded to Gem+SIBI+Rapa treatment, but eventually relapsed under this specific treatment regimen. Intriguingly, using the PEG-Gem+SIBI+Rapa combination we not only observed virtually complete tumour regression, but most importantly we obtained 100% survival throughout the 125d study period in this highly therapy-resistant tumour. While

this observation does not exclude later re-growth as seen in PDAC-Pax22, these data are very promising and are consistent with the notion that further improving the formulation of the combined drugs is mandatory for extending *in vitro* findings to the much more complex *in vivo* setting.

The utilized new smoothened inhibitor SIBI-C1 was also highly effective *in vivo*, as can be seen by reduced tumour growth in combination with Gem and thus significantly enhanced survival time. Furthermore, SIBI can be safely administered *in vivo*, as we saw no adverse effects on total body weight or white blood cell counts. Importantly, this is well in line with previous observations using other smoothened inhibitors (Mueller et al., 2009, Lonardo et al., 2010). The Gem+SIBI+Rapa combination therapy also showed no significant toxicity compared to Gem treatment alone during the course of the experiments. PEG-Gem+SIBI+Rapa combination treatment, while much more effective *in vivo*, only slightly, but non-significantly decreased white blood cell counts and had no effect on the body weight of the animals as compared to respective controls. It is important to note, however, that the healthy and relatively young mice used for this study are likely more capable of compensating for putative adverse effects on the normal stem cell compartments during triple therapy treatment. Therefore, it will be important to further validate the safety of this treatment regimen in human patients in order to ultimately apply it to the mostly aged and moribund patients suffering from pancreatic cancer.

Perspective

The high resistance of cancer stem cells against established standard therapies has received more and more attention since being described in solid tumours such as glioblastoma (Bao et al., 2006), pancreatic (Hermann et al., 2007), and colorectal cancer (Todaro et al., 2007). Since these cells will survive therapy and possibly re-generate the entire tumour after primarily successful treatment in a process called tumour relapse, finding new and specific ways to target and eliminate these cells will be of paramount importance for the long-term success of cancer therapy.

In the projects performed within the scope of the presented doctoral thesis, we have proposed and evaluated three novel therapeutic approaches for the treatment and targeted elimination of cancer stem cells in colorectal and pancreatic cancer. We have

identified pathways (mTOR, hedgehog, Nodal/Activin), and cellular properties (high DNA-damage response mediated by ATR) that are highly typical for (cancer) stem cells. Targeting these stem cell features should then either kill cancer stem cells directly by inhibition of an essential pathway, or affect them in a way that they are then accessible to treatment with chemotherapy. This could happen by either driving CSCs into differentiation, or by pushing usually quiescent cancer stem cell populations into an active cell cycle, making these proliferating cells an easy target for chemotherapeutic agents. In all of the three mentioned strategies, chemotherapy will still play an integral part, killing differentiated cancer cells, as well as proliferating CSCs. Thus a multimodal therapy including CSC-targeting agents as well as chemotherapy or radiation will be necessary for successful therapy. Without a doubt, there will most likely be no “one approach fits all” treatment. Considering the vast heterogeneity between different patient tumors, it rather very likely that CSCs from different tumors will respond in very distinct ways to therapeutic approaches. Therefore, it will be of paramount importance to identify further mechanisms that CSCs use for their survival and propagation. This will eventually enable us to tailor treatments specifically to the needs and a predicted response profile of individual patients.

Just recently, a combination therapy comprising a hedgehog antagonist (Infinity’s IPI-926) and gemcitabine as chemotherapeutic agent has failed and led to the premature termination of a phase III clinical study in metastatic pancreatic cancer. Interestingly, IPI-926 (or Saridegib) is an inhibitor of Smoothened, and not of the downstream effectors of the Gli protein family. Since an activating mutation in K-Ras is one of the most frequent genetic alterations found in PDAC, and K-Ras activates Gli effector proteins, inhibition of upstream smoothened most likely is not sufficient for successful inhibition of the hedgehog pathway in cancer stem cells. However, a surprising finding of this trial is that the survival of gemcitabine + Saridegib-treated patients was even worse than that of patients receiving gemcitabine only. While this is surprising at first sight, a potential explanation for this observation is that the addition of the Smoothened inhibitor to chemotherapy resulted in at least partially successful elimination of the stroma, leading to a “cytokine storm”, i.e. the massed release of pro-inflammatory cytokines from the tumor stroma.

In summary, there is increasing evidence that multimodal therapies will be necessary for the successful treatment of gastrointestinal tumors, and that the elimination of cancer stem cells will play an integral part in the success or failure of any therapeutic approach. Taking this into account, we identified three different new therapeutic approaches, and indeed observed that multimodal therapy resulted in a great therapeutic benefit: only in mice treated with the different combination therapies, cancer stem cells were completely abrogated, and we could witness a long-term disease stabilization or regression, and subsequent long-term survival.

Thus we here provide compelling evidence for the efficacy of multimodal therapies targeting CSCs as well as differentiated cells in colorectal and pancreatic cancer. Taken together, these findings should significantly impact the future development of new therapies for colorectal and pancreatic cancer, and may offer a new step on the path towards curing these deadly diseases.

CONCLUSIONS

1. We have successfully verified the importance of cancer stem cells for the initiation, but also for the propagation of tumours in pancreatic and colorectal cancers.

2. With the DNA damage response kinase ATR (Ataxia Telangiectasia- and Rad3-Related), and the developmental Nodal/Activin signaling pathway we have identified two novel targets for the elimination of cancer stem cells, especially when used in combination with chemotherapy.

3. We demonstrate that chemical inhibition of ATR or inhibition of its direct downstream target Chk1 with small molecule inhibitors or genetic targeting leads to an elimination of cancer stem cells *in vitro*, as evidenced in abrogated sphere formation and dramatically reduced expression of CD133.

4. More importantly, we demonstrate that pre-treatment of primary patient-derived cancer cells *in vitro* dramatically decreases *in vivo* tumourigenicity of these cells, the key readout for successful elimination of cancer stem cells. This decreased tumourigenicity *in vivo* translated into significantly enhanced long-term survival of mice.

5. Chemical and genetic interference with the Nodal/Activin pathway eliminates CSCs as shown by expression levels of stemness-associated genes, reduced CSC markers such as CD133 or CD44, virtually completely abrogated sphere formation, and, most importantly, dramatically reduced tumourigenicity *in vivo.*
   This effect is specific for Nodal/Activin, since chemical inhibition of TGF-b shows no effects, but gene knockdown for Alk4 had the same functional consequence as Nodal/Activin inhibition with small molecule inhibitors.

6. The successful elimination of CSCs *in vitro* through inhibition of the Nodal/Activin pathway was reproducible in a clinically highly relevant *in vivo* model of pancreatic cancer using patient-derived tumour tissue. Combination therapy with pathway inhibitors and standard chemotherapy resulted in significantly reduced tumour size, but, more importantly, in significantly extended overall survival of mice.

7. Co-administration of inhibitors of the sonic hedghehog pathway dramatically increased drug delivery to and drug concentration in the tumour tissue. This led to long-term stable disease, and subsequently to long-term overall survival in treated mice.

8. While Gemcitabine has a limited circulation time and low penetration into tumour tissue *in vivo*, in an attempt to optimize chemotherapy we have shown that applying Gemcitabine bound to poly-ethylene glycol (PEG Gem) can significantly enhance survival time in combination with cancer stem cell-targeting agents.

9. Finally we have reproduced our previous findings, that cancer stem cells can be eliminated using a combination of sonic hedgehog inhibition, mTOR inhibition, and chemotherapy.

CONCLUSIONES

1. Hemos verificado exitosamente la importancia de las células troncales cancerígenas en la iniciación, pero también, en la propagación de los tumoures pancreáticos y colorectales.

2. Con la respuesta al daño del DNA de la Kinasa (Ataxia Telangiectasia- and Rad3-Related), y el desarrollo de la ruta de señalización Nodal/Activin , hemos identificado dos nuevas dianas para la eliminación de las células troncales cancerígenas, especialmente cuando se usan en combinación con quimioterapia.

3. Hemos demostrado que la inhibición química de ATR o la inhibición de su proteína diana Chk1 con una pequeña molécula inhibidora o con una diana genética conlleva a la eliminación de las células troncales *in vitro*, observándose una reducción total en la formación de esferas y una reducción dramática de la expresión del marcador CD133.

4. Mas importante, hemos demostrado que el pre-tratamiento de las células cancerígenas primarias derivadas de paciente *in vitro,* dramáticamente reduce la tumourigenicidad *in vivo* de estas células, prueba clave en el éxito de la completa eliminación de las células troncales cancerígenas. Este descenso de tumourigenicidad *in vivo* se traduce en un significante aumento de la supervivencia a largo plazo de los ratones.

5. La interferencia genética y química con la ruta Nodal/Activin elimina las células troncales cancerígenas tal y como es mostrado en la expresión de los niveles de los genes estaminales asociados, como la reducción de los marcadores CD133 o CD44, la eliminación completa de la formación de esferas y lo mas importante, la dramática reducción de la tumourigenicidad *in vivo.*
   Este efecto es especifico para la ruta Nodal/Activin debido a que la inhibición química de TGF-b no muestra efectos, pero la inhibición de la expresión del gen Alk4 tuvo la misma consecuencia funcional que la inhibición de Nodal/Activin con inhibidores moleculares pequeños.

6. La exitosa eliminación de las células troncales cancerígenas *in vitro* a través de la inhibición de la ruta Nodal/Activin fue reproducible con relevancia clínica en modelos

de cáncer de páncreas *in vivo* utilizando muestras de paciente. La combinación de terapias con los inhibidores de ruta y la quimioterapia estándar, resulto en una reducción significante del tamaño del tumour, pero, aun mas importante, en el aumento significante de la supervivencia global de los ratones.

7. La coadministración de los inhibidores de la ruta de sonic hedghehog dramáticamente aumenta la entrega y también la concentración en el tejido tumoural, conduciendo a una estabilidad a largo plazo de la enfermedad y consecuentemente, a un amuento de la supervivencia global en los ratones tratados.

8. El uso de Gemcitabina ha mostrado que la circulación y penetración en el tejido tumoural *in vivo* es limitada, por tanto, en un intento de optimizar la quimioterapia, hemos mostrado que aplicando Gemcitabina ligada a un polyetilenglicol (PEG Gem) puede significantemente aumentar el tiempo de supervivencia en combinación con terapias diana contra las células troncales cancerígenas.

9. Finalmente, hemos reproducido nuestros descubrimientos anteriores en que las células troncales cancerígenas pueden ser eliminadas usando una combinación de inhibidores de sonic hedgehog y mTOR con quimioterapia.

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